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# THE EQUILIBRIUM BETWEEN OXYGEN AND HEMOGLOBIN IN CONCENTRATED UREA SOLUTION

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(Received for publication, March 3, 1942)

The dissociation of the horse hemoglobin molecule into 2 half molecules in 4 M urea solutions has been well established (16). The effect of concentrated urea on the equilibrium between oxygen and hemoglobin has, however, not been determined. The present experiments constitute a study of this equilibrium.

The reaction between oxygen and hemoglobin is best described if it is assumed that 4 molecules of oxygen react successively with a single molecule of hemoglobin (Hb<sub>4</sub>) containing four heme groups. This assumption leads to the equations of Adair (1), of Ferry and Green (8), and of Pauling (14), all of the same general type; Pauling's equation has but two constants.

Between oxygen saturations of 10 and 90 per cent, the reaction of oxygen with hemoglobin, under the conditions existing in blood, may be described approximately by the simpler equation of Hill (11),

$$\frac{[\text{Hb}]_n \times [\text{O}_2]^n}{[\text{HbO}_2]_n} = K$$

in which  $n^1 = 2.43$ ,  $K = 2.5 \times 10^3$ , for whole blood at 37°, pH 7.4 (10).

Coryell (6) has pointed out that it is the stepwise addition of oxygen to the four heme groups (or, in Pauling's sense, the interaction among them) which accounts for the appearance of non-integral values of  $n$  less than 4 in the above equation. In fact, Coryell has shown that the value of  $n$ , over the central portion of the curve, yields an indication of the degree of the heme-heme interaction.

Theorell (21) and Hill (12) have shown that the oxygenation of myoglobin proceeds according to the above equation with  $n = 1$ . Myoglobin has but one, or possibly two, heme groups in a single molecule (15, 17, 20).

Burk and Greenberg (4, 5), Wu and Yang (24), and Steinhardt (16) have demonstrated that hemoglobin of the horse, of molecular weight

\* National Research Council Fellow in Medicine, 1937-39, during which time these experiments were begun.

<sup>1</sup> The symbol  $n$  in the above equation should not be confused with that used in the classical electrode equation. In the latter  $n$  specifically denotes the number of electrons involved in the electrode reaction; in the former it is an empirical constant.



68,000, containing four heme groups, possesses half that molecular weight in aqueous 4 M urea solution. The "half molecules" retain their characteristic spectral properties and unite with the same total amounts of oxygen or carbon monoxide. They are less stable, however, and gradually become altered ((16), Drabkin (7)). It has been assumed that each half molecule, in urea, contains two of the four heme groups of the original hemoglobin molecule. Taylor (18) has observed that methemoglobin of the horse in 4 M urea is reduced to hemoglobin with the apparently simultaneous addition of 2 electrons.

We have studied the equilibrium between oxygen and hemoglobin of the horse in aqueous 4 M urea, at approximately the isoelectric point of the hemoglobin, in order to determine the value of  $n$ , in Hill's equation, and to observe whether the value so determined may be correlated with dissociation of the hemoglobin molecule in urea.

#### EXPERIMENTAL

All the experiments to be reported were performed upon a single preparation of horse oxyhemoglobin twice recrystallized as previously described (19). The crystals were thrice washed with cold distilled water saturated with toluene, and were preserved at 4° under an atmosphere saturated with toluene. This method has been found to preserve the oxyhemoglobin preparations for many weeks.

Urea was twice recrystallized. A weighed quantity of the moist oxyhemoglobin crystals was dissolved in enough 5.75 M urea solution to bring the volume to 100 ml. The quantities were such that the final urea concentration, calculated from the weights taken, approximated 4 M. In order to avoid the effects of added salts, no buffers were added, but the pH of the solutions was determined electrometrically as described below.

The hemoglobin-urea solutions were equilibrated at varying oxygen tensions for 30 minutes at 37° by the "first saturation method" of Austin *et al.* (3) with the double tonometer technique. The gas and liquid phases were then separated and analyzed. Oxygen was determined in the gas phase with the Haldane apparatus, in the liquid phase with the Van Slyke manometric apparatus. But one equilibration was performed at a time, in order that the analyses might be completed as quickly as possible after equilibrium had been established. After the oxygen content of the liquid phase had been determined, a sample was ejected from the tonometer, fully oxygenated by exposure to air, and analyzed for its oxygen capacity.

The pH was determined on a sample from the tonometer, without exposure to air. These measurements were made at room temperature with a MacInnes and Belcher (13) glass electrode connected to a Leeds and Northrup portable pH electrometer sensitive to  $\pm 0.02$  pH. The correc-

tion which was applied to bring the pH of the hemoglobin solutions in 4 M urea, measured at room temperature, to that at 37° was determined in a separate experiment. No corrections have been applied for the liquid junction between saturated KCl and 4 M urea.

The process of equilibration by rotation of the tonometer at 37° produced extensive precipitation of the hemoglobin in the presence of urea. As estimated by comparison of the oxygen capacities before and after rotation, this amounted to 20 per cent of the total hemoglobin in some instances. It was also found that the oxygen capacity decreased and a

TABLE I  
*Solubility of Oxygen in Water and in 4 M Urea Solution*

$A = 50$  ml.,  $S = 20$  ml.,  $a = 2.0$  ml.,  $K_1 = 10$ ,  $K_2 = 0.664$  at 25°, 0.663 at 37°,  $i = 1.00$ . The symbols are those used by Van Slyke (22), and are mentioned as an indication of the conditions that we used.

Liquid	Temperature	$\alpha$	$\alpha$ (Winkler (23))	$\alpha$ (Fox (9))
	°C.			
Water	23.4	0.0295	0.0291	0.0297
	23.6	0.0293	0.0290	0.0296
	24.0	0.0293	0.0288	0.0294
	38.0	0.0238	0.0235	0.0240
	37.0	0.0241	0.0238	0.0243
	37.2	0.0239	0.0238	0.0242
4 M urea	24.4	0.0216		
	24.7	0.0216		
	37.3	0.0187		
	37.1	0.0193		
	37.0	0.0192		
	37.2	0.0193		

slight precipitate gradually appeared in stock solutions of hemoglobin in 4 M urea kept at 4° without rotation. This phenomenon may represent the secondary process of denaturation, following the splitting in urea, mentioned by Steinhardt (16) and by Drabkin (7).

*Correction for Dissolved Oxygen*—We have determined the solubility of oxygen in 4 M urea at room temperature and at 37° by Van Slyke's manometric method (22). The results appear in Table I.  $\alpha$  is the Bunsen solubility coefficient.

The dissolved oxygen values computed from these solubility coefficients have been subtracted from the results of the oxygen analyses on the liquid samples to yield values for oxyhemoglobin.

## Results

The data of two experiments in the presence of urea have been plotted graphically in Fig. 1. The curve through the experimental points satisfies the equation

$$\frac{[\text{Hb}]_{1.5} \times [\text{pO}_2]^{2.9}}{[\text{HbO}_2]_{1.5}} = 10^{1.23} \quad (1)$$

The oxygen dissociation curve of oxyhemoglobin in water at pH 6.60 and

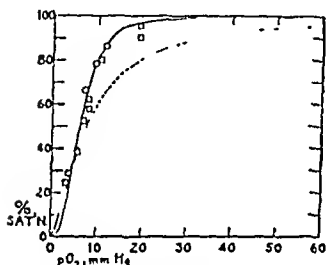


FIG. 1

FIG. 1. Relation of per cent saturation of hemoglobin to oxygen pressure.  $\circ$  Experiment A,  $\square$  Experiment C, in 4 M urea at  $37^\circ$ ; the solid line represents Equation 1. The dotted line represents Equation 2, describing the behavior in the absence of urea (2).

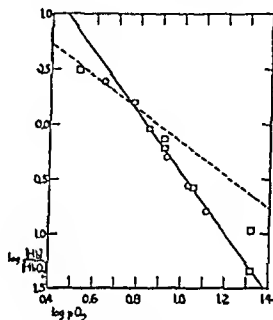


FIG. 2

FIG. 2. Relation of  $\log [\text{Hb}]/[\text{HbO}_2]$  to  $\log \text{pO}_2$ .  $\circ$  Experiment A,  $\square$  Experiment C, in 4 M urea at  $37^\circ$ , corrected to pH 6.6 as described in the text. The solid line represents Equation 3; the dotted line represents Equation 4.

$37^\circ$  estimated from the data of Altshul and Hogness (2) is also given for reference. This curve, in the absence of urea, satisfies the equation

$$\frac{[\text{Hb}]_{1.5} \times [\text{pO}_2]^{1.5}}{[\text{HbO}_2]_{1.5}} = 10^{1.22} \quad (2)$$

The degree to which urea affects the equilibrium between oxygen and hemoglobin is more clearly seen in Fig. 2 by comparing the logarithmic plots of the two equations,

$$\log [\text{Hb}]_{1.5} - \log [\text{HbO}_2]_{1.5} + 2.9 \log \text{pO}_2 = 2.28 \text{ (with urea)} \quad (3)$$

$$\log [\text{Hb}]_{1.5} - \log [\text{HbO}_2]_{1.5} + 1.5 \log \text{pO}_2 = 1.32 \text{ (without urea)} \quad (4)$$

The experimental points in this case have been corrected to the oxygen tensions with which they would have been in equilibrium at the same oxygen saturation, had the equilibrations been carried out at exactly

pH 6.60. This correction was made by assuming that the value of  $n$  does not vary with pH and that the value of  $\log K$  varies according to the relation,  $\Delta \log K / \Delta \text{pH} = 0.81$ , calculated from the data of Altschul and Hogness (2) for hemoglobin in water.

It is seen at once that most of the experimental points deviate widely from the line representing the equilibrium of hemoglobin with oxygen in the absence of urea; furthermore, that all points, save one, lie close to the line representing Equation 3. Whether or not the deviation of the points at the lowest oxygen tensions is real or fortuitous, it is not possible to say on the basis of the available data.

Another mathematical formulation showing a closer relation to Equation 4 fits our data in 4 M urea equally well; namely,

$$\log [\text{Hb}]_{29} - 2 \log [\text{HbO}_2]_{29} + 3 \log p\text{O}_2 = 2.26 \quad (5)$$

At the present time, Equations 3 and 5 must be regarded as empirical and a choice as to which is preferable cannot be made.

### Comment

The data show that for a given oxygen tension a greater amount of oxygen is combined with hemoglobin in the presence of urea than in its absence. Whether this is the direct effect of the dissociation of hemoglobin molecules having a molecular weight of 68,000 into those having a molecular weight of 34,000, or whether it is due to an indirect influence of urea on the combination of oxygen with hemoglobin, cannot be stated. It is significant, however, that myoglobin, with the same prosthetic group and with a molecular weight of 17,000 (15, 17) or possibly 34,000 (20), likewise becomes oxygenated at lower oxygen pressures than does hemoglobin (12, 21).

As previous studies of the oxygenation of hemoglobin in the absence of urea have shown, there is no direct correlation between the molecular weight of hemoglobin and the value of  $n$ . While a correlation appears between the low molecular weight of myoglobin and the observed value of  $n = 1$  (12, 21), the results of the present investigation imply a negative correlation between the value of  $n$  and the lowered molecular weight of hemoglobin in 4 M urea solution.

### SUMMARY

The equilibrium between oxygen and horse hemoglobin in 4 M urea solution has been investigated.

At a given oxygen pressure, pH 6.6 and 37°, hemoglobin in 4 M urea solution combines with more oxygen than in the absence of urea.

The equation  $([\text{Hb}]_{29} \times [p\text{O}_2]^{2.9}) / [\text{HbO}_2]_{29} = 2.28$  has been found to describe the data.

The lack of correlation, in 4 M urea solution, between the value of  $n$  and the molecular weight of hemoglobin has been pointed out.

The solubility of oxygen in 4 M urea solution has been determined:  $\alpha = 0.0216$  at  $24.5^\circ$ ,  $\alpha = 0.0192$  at  $37.0^\circ$ .

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# OXIDATION-REDUCTION POTENTIALS OF THE METHEMOGLOBIN-HEMOGLOBIN SYSTEM IN CONCENTRATED UREA SOLUTION\*

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The molecular weight of hemoglobin, of the horse or ox, dissolved in a sufficiently concentrated aqueous solution of urea, or other amide, has been found to be 34,000, or one-half that found in the absence of amide (1, 2, 15, 18). Steinhardt has established that the dissociation into half weight molecules is complete in 4 M urea. He has also determined that the oxygen and carbon monoxide capacity and the spectroscopic characteristics of hemoglobin are not altered in urea. The half molecules are less stable, however, and may easily become denatured (9, 15). It is not known whether 1 hemoglobin molecule yields 2 half molecules that are structurally identical with respect to their protein portions, or with respect to the number of heme prosthetic groups.

Although the methemoglobin-hemoglobin system is thermodynamically reversible, the oxidation-reduction potentials that are observed, as methemoglobin is reduced in aqueous phosphate buffers, do not follow the course described by the electrode equation

$$E_A = E_m' + \frac{RT}{nF} \ln \frac{[\text{methemoglobin (Fe}^{+++}\text{)}]}{[\text{hemoglobin (Fe}^{++}\text{)}]} \quad (1)$$

As hemoglobin is oxidized to methemoglobin, the slope of the titration curve varies progressively from that corresponding to Equation 1 with  $n = 1$  toward that for  $n = 2$  (17). Although a satisfactory explanation of this behavior has not yet been given, it has been suggested that interactions among the four hemes are responsible (7, 17).

It is the purpose of the present communication to report the oxidation-reduction potentials, at varying pH values, of methemoglobin-hemoglobin mixtures in aqueous 4 M urea. We have found that the oxidation-reduction reaction is described by Equation 1 with  $n = 2$ .

## EXPERIMENTAL

Crystalline oxyhemoglobin of the horse was prepared as previously described (17). Oxyhemoglobin was also crystallized from dog blood by

\* A preliminary report of this work was presented before the Thirty-third annual meeting of the American Society of Biological Chemists at Toronto, 1939 (16).

† National Research Council Fellow in Medicine, 1937-39, during which time these experiments were begun.

the same procedure. The crystals were thrice washed with cold distilled water saturated with toluene, and were preserved at  $4^{\circ}$  under an atmosphere saturated with toluene vapor. This method has been found to preserve the oxyhemoglobin preparations for many weeks.

Urea was twice recrystallized. In order to study the oxidation-reduction potentials from pH 6.2 to 7.6, stock phosphate buffers were prepared from the tables of Green (12), but of twice the concentration necessary to give, on dilution, a final ionic strength of 0.2. Other buffers were not employed, since the pH limits of the phosphate series proved to be adequate for the range in which stable electrode potentials were obtained. The stock buffers were diluted to twice their original volume with water, or with water and sufficient urea to bring the urea concentration to 4 M. The pH values of the diluted buffers were measured at  $30^{\circ}$  (a) with a Clark hydrogen electrode (3), and (b) with a glass electrode of the type introduced by MacInnes and Belcher (13), connected to a vacuum tube amplifier similar to that described by van Dyke and Bennett (10). No correction was applied for the liquid junction between saturated KCl and 4 M urea. The pH values obtained with the two methods agreed to within 0.01 pH unit. The pH of urea-buffer mixtures containing hemoglobin was, therefore, measured by means of the glass electrode at the end of each titration.

Solutions for titration were prepared by the following procedure. Approximately 7 gm. of moist oxyhemoglobin crystals were dissolved in the stock, double strength buffer to make approximately 70 ml. Undissolved material was removed. (This concentration is near the limit of solubility, especially for dog oxyhemoglobin in the neighborhood of its isoelectric point.) Kjeldahl analyses for nitrogen were performed upon 2 ml. aliquots of this solution. The nitrogen analyses were converted into hemoglobin concentrations, 17.7 per cent being used as the nitrogen content of horse hemoglobin (11). 50 ml. of the hemoglobin solution and 24.02 gm. of urea, dissolved in the minimal amount of water, were mixed with care and diluted to 100 ml. 50 ml. of this solution were placed in the titration cell. The hemoglobin was oxidized to methemoglobin with  $K_3Fe(CN)_6$  and titrated back to hemoglobin with reduced anthraquinone- $\beta$ -sulfonate. The technical details of the titration procedure were the same as those previously described (17). All measurements were performed at  $30^{\circ}$ . The electrode potentials, as well as the pH values, have been referred to the normal hydrogen electrode, after the conventions of Clark (3). (This corresponds to assigning the value 4.62 to the pH of standard acetate, at  $30^{\circ}$ .) As in the measurement of pH, liquid junctions were made against saturated KCl containing no urea, with no corrections for the presence of urea.

Toluylene blue proved to be a suitable mediator and was, therefore,

used in the experiments on urea-containing solutions. A few orienting titrations of toluylene blue and *m*-tolylenediamine indophenol were performed in 4 M urea-buffer mixtures. Over the pH range 6 to 7, their titration curves were displaced approximately 0.015 volt toward more negative potentials.

TABLE I  
*Relation of  $E_h$  to Per Cent Reduction*

Titration of horse methemoglobin with reduced anthraquinone- $\beta$ -sulfonate at constant pH in 4 M urea. Demonstration that  $n = 2$ . Hemoglobin 15.5 gm. per liter; urea = 4.0 M; toluylene blue = 0.00002 M; pH = 7.67; temperature = 30°;  $d = 0.364$  ml.; 100 per cent reduction at  $y = 1.511$  ml.;  $y$  and  $d$  have the conventional meanings as used by Clark *et al.* (4).

$y$	$y-d$	Reduction	$0.03055 \times \log \frac{S_a}{S_r}$	$E_h$	$E'_m$	Deviation from +0.0718
ml.	ml.	per cent	volt	volt	volt	volt
0.40	0.036	2.41	+0.0181	+0.1184	(+0.0703)	-0.0015
0.50	0.136	9.21	+0.0299	+0.1025	(+0.0726)	+0.0008
0.60	0.236	15.98	+0.0217	+0.0938	+0.0721	+0.0003
0.70	0.336	22.75	+0.0160	+0.0876	+0.0716	-0.0002
0.80	0.436	29.52	+0.0114	+0.0828	+0.0714	-0.0004
1.00	0.636	43.06	+0.0037	+0.0756	+0.0719	+0.0001
1.10	0.736	49.81	+0.0001	+0.0718	+0.0717	-0.0001
1.20	0.836	56.61	-0.0035	+0.0684	+0.0719	+0.0001
1.30	0.936	63.38	-0.0072	+0.0647	+0.0719	+0.0001
1.40	1.036	70.15	-0.0112	+0.0608	+0.0720	+0.0002
1.50	1.136	76.92	-0.0157	+0.0561	+0.0718	0.0000
1.60	1.236	83.69	-0.0213	+0.0507	+0.0720	+0.0002
1.72	1.356	91.81	-0.0315	+0.0420	(+0.0735)	+0.0017
1.80	1.436	97.23	-0.0464	+0.0315	(+0.0779)	+0.0061
Average .....					+0.0718	

### Results

*Titration Curves of Hemoglobin in 4 M Urea*—Complete titration curves in 4 M urea, determined by approximately fifteen points each, have been carried out on seven solutions of horse hemoglobin and three solutions of dog hemoglobin.

A typical example of the reduction of horse methemoglobin by reduced anthraquinone- $\beta$ -sulfonate at pH 7.67 in 4 M urea is presented in detail in Table I. That the data are described by Equation 1, with  $n = 2$ , is shown by the constancy in the values of  $E'_m$  calculated from this equation. Rectification of the titration curve by the objective method of Reed and Berkson (14, 4) also indicated that  $n = 2$ . Application of this method to



all the titration curves showed that  $n$  equals 2 for both horse and dog hemoglobin in 4 M urea.

The values of  $E'_m$  at various pH values, calculated according to Equation 1 with  $n = 2$  by the aid of the Reed and Berkson method, are given in Table II. The values for dog hemoglobin are seen to be not more than 0.005 volt more negative than those for horse hemoglobin, at corresponding pH values.

*Titration Curves of Dog Hemoglobin in the Absence of Urea*—The results of two experiments on dog hemoglobin, in the absence of urea, are also included in Table II. The  $E'_m$  values of these experiments are about

TABLE II

*Methemoglobin-Hemoglobin, Relation of  $E'_m$  to pH at 30°*

Phosphate buffers, ionic strength = 0.2; mediator, toluylene blue.

Species	Urea	pH	$E'_m$
	M		volt
Horse	4	6.23	+0.1216
	4	6.23	+0.1206
	4	6.61	+0.1189
	4	6.94	+0.1101
	4	7.32	+0.0942
	4	7.58	+0.0702
	4	7.67	+0.0718*
	4	6.22	+0.1211
Dog	4	6.92	+0.1060†
	4	7.30	+0.0892
	0	5.85	+0.1535
	0	7.10	+0.1318‡

\* Reported in detail in Table I.

† Agreement with  $n = 2$  not as satisfactory as in other instances.

‡ Mediator *m*-toluylenediamine indophenol.

0.010 volt more negative than those previously determined for horse hemoglobin at the same pH values (17). The slope of the titration curve of dog hemoglobin is indistinguishable, by the graphical methods that are applicable, from that of similar curves for horse hemoglobin (17), and likewise indicates a progressive variation in  $n$  from 2 to 1 as methemoglobin is reduced to hemoglobin.

#### DISCUSSION

The values of  $E'_m$  obtained for horse and for dog hemoglobin, in the absence and presence of urea, are plotted against pH in Fig. 1.

The oxidation-reduction potential of a mixture of methemoglobin and

hemoglobin of the horse becomes more negative as the pH of the solution increases beyond 6.0. To describe the data in the absence of urea, Taylor and Hastings (17) proposed an equation involving a single acidic dissociation constant in the oxidant at  $2.2 \times 10^{-7}$  ( $pK = 6.65$ ). Coryell and Pauling (8) suggested that the change of  $E'_m$  with pH might be the resultant effect of several dissociable groups in the oxidant and reductant. Wyman and Ingalls (19) have recently determined the significant acid-base dissociation constants of methemoglobin and hemoglobin, from which they have constructed a curve relating  $E'_m$  and pH, and which they have fitted to the data of Taylor and Hastings (17) (see Fig. 1).

The curve of Wyman and Ingalls yields a value of +0.144 volt at pH 7.0.<sup>1</sup> This is believed to be a more accurate value than that estimated by

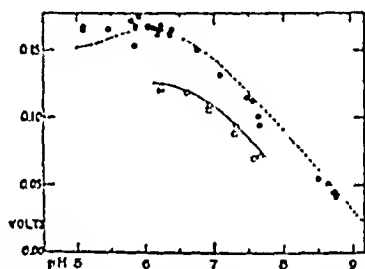


FIG. 1. Relation of  $E'_m$  to pH.  $E'_m$  is the potential of the system at 50 per cent reduction. Hemoglobin, O of the horse, □ of the dog, in 4 M urea solution (Table II); the solid line is drawn as described in the text. Hemoglobin, ● of the horse (17), ■ of the dog (Table II), in the absence of urea. The dotted line is drawn according to Wyman and Ingalls (19).

Taylor and Hastings from the curve suggested by them (17). The agreement with Conant and Pappenheimer (5), +0.152 volt at pH 7.0 (potentiometric), and with Conant and Scott (6), +0.147 volt, corrected to pH 7.0 (spectrophotometric), is also more satisfactory.

The data of the experiments in 4 M urea may also be fitted fairly closely by the curve of Wyman and Ingalls by displacing the latter +0.1 pH unit and -0.041 volt, shown in Fig. 1 by the solid line. Although the data of the experiments in 4 M urea are inadequate for a rigorous mathematical treatment, it would appear from the shape and position of the curves in Fig. 1 that the same acidic and basic groups are involved in determining the variation of  $E'_m$  with pH in the presence of urea as in its absence. The absolute values of both pH and  $E'_m$  are subject to uncertainties with respect

<sup>1</sup> In this laboratory, Mr. Felix Heimberg has recently titrated horse methemoglobin at pH 7.0. His values at an ionic strength of 0.2 average +0.149 volt  $\pm 0.002$ .

to the effect of strong urea solutions upon the dissociation constants and activity coefficients of the molecular species present.

Subject to these uncertainties, the  $E'_m$  of the methemoglobin-hemoglobin system in 4 M urea is +0.108 volt at pH 7.0.

Since the value of  $n$  is 2, it might be concluded that the four heme groups present in hemoglobin have separated equally, two going to each half weight molecule in 4 M urea. If these two groups become oxidized or reduced "at the same time" (*i.e.*, not stepwise), the value of  $n$  should equal 2. Although this explanation would be adequate to explain our results in 4 M urea, it sheds no light on the reason for the low and progressively changing values of  $n$  (from 1 to 2) in the absence of urea.

It should be noted that our determinations of the value of  $n$  for dog hemoglobin agree with the results for horse hemoglobin, both in the presence and absence of urea. The shift in values of  $E'_m$  in 4 M urea is also essentially the same in both instances. From osmotic pressure measurements, Wu and Yang (18) have reported that although horse and ox hemoglobins dissociate in urea solution, the hemoglobins of sheep and dog do not. This may mean that the changes produced in the values of  $n$  and  $E'_m$  in 4 M urea are the result of factors other than the dissociation of hemoglobin into half molecules. It would appear desirable to restudy the question of the dissociation of dog hemoglobin in urea with the ultracentrifuge technique.

#### SUMMARY

The oxidation-reduction potentials of the methemoglobin-hemoglobin system have been studied in aqueous 4 M urea solutions containing phosphate buffer mixtures.

The oxidation-reduction reaction in urea appears to involve the transfer of 2 electrons in the oxidation of both horse and dog hemoglobin.

The potential of an equimolar mixture of horse methemoglobin and hemoglobin in 4 M urea has been found to be +0.108 volt at 30° at pH 7. The comparable value in the absence of urea has been newly estimated as +0.144 volt.

The mid-point potential varies with pH in a manner essentially parallel with the behavior of the same system in the absence of urea.

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pH of 6.6. This procedure had the advantage of keeping the solutions concentrated. An appreciable quantity of brown crystalline material, presumed from its solubility (9) and crystalline form (7) to be methemoglobin, was then removed by filtration. Phosphate was removed by dialysis against distilled water, yielding stock solutions containing metmyoglobin in a concentration of approximately 2 per cent. These were used directly in the preparations of solutions to be titrated.

Spectroscopic observation indicated that the myoglobin was converted to metmyoglobin during the course of the preparation. A small amount of ferrieyanide was added, before the reductive titrations were commenced, to insure complete oxidation of the myoglobin. The concentration of metmyoglobin in the stock solutions was estimated by spectrophotometric observations at a wave-length of 630 m $\mu$ , in phosphate buffer at pH 7.0, the extinction coefficient determined by Theorell (19) being used.

In order to study the oxidation-reduction potentials from pH 5.9 to 7.4, stock phosphate buffers were prepared from the tables of Green (5), but of 5 times the concentration necessary to give, on dilution, a final ionic strength of 0.2. The stock buffers were diluted to 5-fold their *original volume, either with the stock metmyoglobin solutions or with water*, to provide the solutions used for the oxidation-reduction titrations and pH measurements. The pH values of the myoglobin-buffer mixtures were measured at the end of each titration with the use of the glass electrode previously described (15). The electrode potentials and pH values have been referred to the normal hydrogen electrode after the conventions of Clark (3). (This corresponds to assigning the value 4.62 to the pH of standard acetate, at 30°.)

Metmyoglobin solutions prepared as described above were titrated with reduced anthraquinone- $\beta$ -sulfonate by the technique and with the apparatus previously described (16). Toluylene blue and cresyl blue were used as mediators. Except at the ends of the curves, stable potentials without drifting were obtained within less than 10 minutes, and over fifteen points were obtained in the course of each titration, thus permitting accurate characterization of the curves.

In early experiments performed by one of us (V. E. M.), the oxidation-reduction potentials of metmyoglobin-myoglobin mixtures in the absence of mediators were found to exhibit the sluggish behavior characteristic of hemoglobin systems (16). It was necessary under these conditions to wait from 1 to 2 hours after each addition of oxidant or reductant for the establishment of even apparent equilibrium. This precluded the possibility of obtaining more than a few points in any one titration. In many instances lack of agreement between electrode potentials and drifting contributed to the uncertainty of the data.

## Results

Table I summarizes the results of a titration in the presence of mediators at pH 6.95. The values for  $E'_m$  given in the sixth column have been calculated for a transfer of 1 electron during the titration according to Equation 1. The agreement is seen to be satisfactory. Analysis of all

TABLE I  
Relation of  $E_h$  to Per Cent Reduction

Titration of horse metmyoglobin with reduced anthraquinone- $\beta$ -sulfonate at constant pH. Demonstration that  $n = 1$ . Myoglobin 17.0 gm per liter; toluylene blue  $2.5 \times 10^{-4}$  M; cresyl blue  $2.5 \times 10^{-4}$  M, pH 6.95, temperature = 30°;  $d = 0.375$  ml, 100 per cent reduction at  $y = 3.875$  ml,  $y$  and  $d$  have the conventional meanings as used by Clark *et al.* (4)

$y$	$y-d$	Reduction	$0.0011 \times \log \frac{S_o}{S_r}$	$E_h$	$E'_m$	Deviation from +0.0460
ml	ml	per cent	volt	volt	volt	volt
0.50	0.125	3.57	+0.0861	+0.1210	(+0.0349)	-0.0111
0.60	0.225	6.43	+0.0699	+0.1111	(+0.0412)	-0.0048
0.80	0.125	12.14	+0.0517	+0.0966	(+0.0449)	-0.0011
1.00	0.625	17.86	+0.0398	+0.0856	+0.0458	-0.0002
1.20	0.825	23.57	+0.0307	+0.0767	+0.0460	0.0000
1.40	1.025	29.29	+0.0230	+0.0692	+0.0460	0.0000
1.60	1.225	35.00	+0.0162	+0.0623	+0.0461	+0.0001
1.80	1.425	40.71	+0.0098	+0.0559	+0.0461	+0.0001
2.00	1.625	46.43	+0.0037	+0.0498	+0.0461	+0.0001
2.20	1.825	52.11	-0.0022	+0.0438	+0.0460	0.0000
2.40	2.025	57.86	-0.0083	+0.0377	+0.0460	0.0000
2.60	2.225	63.57	-0.0145	+0.0314	+0.0459	-0.0001
2.80	2.425	69.28	-0.0212	+0.0247	+0.0459	-0.0001
3.00	2.625	75.00	-0.0287	+0.0174	+0.0461	+0.0001
3.20	2.825	80.71	-0.0374	+0.0086	+0.0460	0.0000
3.40	3.025	86.43	-0.0483	-0.0019	+0.0464	+0.0004
3.60	3.225	92.14	-0.0643	-0.0165	(+0.0478)	+0.0018
3.70	3.325	95.00	-0.0769	-0.0272	(+0.0497)	+0.0037
3.80	3.425	97.86	-0.0998	-0.0435	(+0.0563)	+0.0103
Average					+0.0460	

titration curves in the presence of mediators, from pH 5.9 to 7.4, by the objective method of Reed and Beikson (4, 10) has shown that 1 electron is involved.

The solution reduced at pH 6.95 was subsequently reoxidized by titration with ferricyanide, yielding data in entire agreement with those given in Table I for the reduction.

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## STUDIES ON THE CAROTENOIDS

### II. THE ISOMERIZATION OF $\beta$ -CAROTENE AND ITS RELATION TO CAROTENE ANALYSIS\*

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Many methods for the determination of the carotene<sup>1</sup> pigments in plant material have been proposed. Most of these are modifications of the original method of Willstätter and Stoll (2). Usually the pigments are extracted from the plant tissue with a solvent, saponified, and distributed between immiscible solvents. The final "carotene" solution is then analyzed by comparison of its light-absorbing properties for a specified wave band with those of a carotene standard. In such comparative methods of pigment determination it has been assumed that all the pigment in the final petroleum ether solution is  $\beta$ -carotene. Generally no accurate allowance is made for the presence of colored impurities. Miller (3) has applied a spectrophotometric method to the analysis of certain plant extracts for  $\beta$ -carotene without separation of chlorophyll and "xanthophyll" pigments. It has been observed that the spectroscopic properties of the carotene of the plant extract and of the  $\beta$ -carotene reference standard do not agree. This has been discussed by Wiseman, Kane, Shinn, and Cary (4), by Peterson, Hughes, and Payne (5), and by Peterson (6).

Various attempts have been made to develop a method by means of which the pigment present in the solution analyzed will be limited to  $\beta$ -carotene. Fraps, Kemmerer, and Greenberg (7) have used selective adsorbents in the preparation of the final petroleum ether solution. Moore (8) has reported the use of filtration of the pigment extract through a short column of dicalcium phosphate. Hegsted, Porter, and Peterson (9) and Zimmerman, Tressler, and Maynard (10) have used aqueous diacetone alcohol for separation of the non-carotene pigments from the solution analyzed.

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Some of the data presented here are from the thesis of B. W. Beadle to be presented to the faculty of Purdue University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

<sup>1</sup> The question of carotenoid nomenclature has been discussed recently by Zscheile (1).



It is the purpose of this paper to show that the carotene fraction from certain fresh plant materials consists of  $\beta$ -carotene plus a pigment which is distinguishable chromatographically from  $\beta$ -carotene and that this fraction from certain plants may be analyzed accurately for both of these pigments. This other pigment is considered to be neo- $\beta$ -carotene,<sup>2</sup> an isomer of  $\beta$ -carotene which has been formed previously from  $\beta$ -carotene in the laboratory (13, 11, 12). This pigment has not been recognized as a naturally occurring substance in plant material,<sup>2</sup> although it has been suggested as a component of butter and of blood serum (11).

Quantitative absorption data are presented for neo- $\beta$ -carotene, making possible the spectrophotometric analysis of a mixture of  $\beta$ -carotene and neo- $\beta$ -carotene for the content of the individual pigments. Plant carotene has been analyzed accurately as a mixture of these pigments, and these findings have been confirmed chromatographically, thus offering a logical explanation for the anomalous absorption spectra of the plant carotene in the instances investigated.

#### EXPERIMENTAL

The method employed in this laboratory for the preparation of carotene fractions from plant tissue is essentially that of Zimmerman, Tressler, and Maynard (10). A few modifications have been made. These are (a) the use of a Waring blender (14) for the extraction process, (b) the use of all solvents, including the diacetone alcohol for the original extraction, at room temperature to eliminate any difficulties due to heating, and (c) the use of a pinch of calcium carbonate during extraction to neutralize any plant acids which might be liberated.

Hexane was the only petroleum solvent used, in order to maintain maximum uniformity of solvent (15). All solvents were purified and redistilled in accordance with the practices of this laboratory (15). The diacetone alcohol was redistilled at 95° under reduced pressure shortly before use.

Absorption measurements were made on a photoelectric spectrophotometer, with a Hilger double monochromator with quartz optics. Slit widths of 0.06 mm. were used. The spectral regions isolated were thus 6.3 to 16 Å. from 3800 to 5000 Å., respectively (15).

10 gm. of fresh spinach leaves were extracted (5 minutes) with 125 ml. of cold (20°) diacetone alcohol in a Waring blender, and the mixture

<sup>2</sup> The term neo- $\beta$ -carotene is used in this paper in preference to pseudo- $\alpha$ -carotene found in the literature (11, 12). Since the parent substance is  $\beta$ -carotene, the term neo- $\beta$ -carotene seems to be more suitable for describing the pigment. Since this paper was prepared, neo- $\beta$ -carotene has been reported by Went, Lerosen, and Zechmeister as a constituent of tomato (*Plant Physiol.*, 17, 91 (1942)).

filtered through paper on a Jena slit sieve filter. The percentage of water in the filtrate was 6.5 by volume. The carotenes were transferred to hexane by four extractions (25 ml. each) in a 500 ml. separatory funnel. The proper concentration of water is important for this transfer. The small amounts of carotenols<sup>1</sup> and chlorophylls in the hexane were removed by three extractions (25 ml. each) with aqueous diacetone alcohol (100 parts of diacetone alcohol plus 7 parts of water by volume), and the last traces of chlorophylls removed by shaking for 1 minute with 25 ml. of 20 per cent potassium hydroxide in methanol at room temperature. The final hexane solution of carotene was washed three times with water and dried over sodium sulfate. The absorption measurements were made at 25 Å. intervals from 3800 to 5000 Å.

The values toward the red were progressively low in relation to those for fresh solutions of pure  $\beta$ -carotene (15) (or the values toward the blue end of the curve were too high). This was observed by plotting the logarithms of the  $\log_{10} I_0/I$  values and comparing the resultant curve with that of a fresh solution of  $\beta$ -carotene in the same solvent. Similar curves were obtained when the sources of pigments were several varieties of spinach, peas, beans, asparagus, Lima beans, and broccoli. Adsorption of the pigment on a column composed of equal parts of magnesium oxide and Hyflo super-cel (15) showed only traces of colored substances other than the main pigment fraction, which was apparently  $\beta$ -carotene. This main pigment fraction was separated from the more strongly adsorbed impurities and eluted by means of 5 per cent ethanol in hexane. After removal of the ethanol with water, the absorption spectrum showed essentially the same characteristics as before the adsorption. The same results were obtained when the more strongly adsorbed impurities were removed in accordance with the method of Fraps, Kemmerer, and Greenberg (7). There seemed to be two possible explanations for the failure of the spectrum of the " $\beta$ -carotene" from plant extracts to agree with that of the highly purified crystalline standard. Either the long process of purification (15) had changed the pigment, so that it differed slightly from naturally occurring  $\beta$ -carotene, or there was some other pigment present in the plant extract which was not distinguishable by the chromatographic method employed. When the carotene solution from spinach was evaporated and crystallized, the crystals were found to be spectroscopically identical with  $\beta$ -carotene, while the mother liquor exhibited spectroscopic properties even more widely different from those of  $\beta$ -carotene than did the original solution.

Consideration was therefore given to the stability of  $\beta$ -carotene under various conditions. Zechmeister and Tuzson (11) and Carter and Gillam (12) have reported that  $\beta$ -carotene can be transformed by heat into pseudo-

$\alpha$ -carotene<sup>2</sup> which has absorption maxima at wave-lengths very close to those of  $\alpha$ -carotene. Therefore, samples of pure  $\beta$ -carotene were refluxed in hexane for periods of time varying from 45 minutes to 48 hours. Adsorption of this heated material on a magnesium oxide column showed the presence of several strongly adsorbed decomposition products, in addition to the principal zone, which was apparently unchanged. This zone was eluted, washed, and found to have an absorption spectrum which was not like that of the original  $\beta$ -carotene, but which resembled that obtained from the carotene fraction of plant extracts in that the absorption values were too low toward the red end of the curve in relation to the values at the blue end. This experiment was repeated several times, with extreme precautions to exclude air, by refluxing under nitrogen passed over hot copper; in one instance, however, pure oxygen was bubbled through the solution during the entire time of refluxing (15 hours). In all experiments, the results were the same. None of the isomer could be distinguished on the columns. At this time, it was also observed that when pure  $\beta$ -carotene was recrystallized the new crystals possessed the same spectroscopic properties in solution as did the original crystals, while the properties of the mother liquor resembled those of the heated solution, although no heat was used during the recrystallization.

The similarity of spectra of these preparations indicated the possibility that the same components were present in all of them. Since the heated solutions should have contained neo- $\beta$ -carotene (11, 12), attempts were made to find an adsorbent which would provide better resolution than did the magnesia. Proper development of an alumina (Merck's anhydrous alumina according to Broekmann) column (11, 12) containing the fractions mentioned above, which appeared homogeneous on magnesia, resulted in the separation of a brownish yellow zone immediately below the  $\beta$ -carotene. The  $\beta$ -carotene fraction which was eluted from this type of column possessed an absorption spectrum which satisfactorily checked that of the reference standard, while the maxima for the other pigment were close to those of  $\alpha$ -carotene, but only in so far as wave-length was concerned and not in regard to relative intensities. The pigment from the lower zone was apparently identical with the pseudo- $\alpha$ -carotene mentioned by Zechmeister and Tuzson (11) and by Carter and Gillam (12). It was found that the carotene fraction from all plant extracts examined which produced a homogeneous zone on magnesia and differed but slightly from the  $\beta$ -carotene reference curve could be resolved into two components on an alumina column. It is unlikely that its presence was due to analytical procedures in this laboratory, since all operations took place at room temperature, and the entire time of preparation of the solution was not more than 1 hour. Solutions of pure  $\beta$ -carotene in hexane

may be kept at room temperature (30°) for 3 hours (1 hour exposed to room light followed by 2 hours in darkness) without great change in the absorption spectrum. At 5° in darkness no significant change was observed at the end of 60 hours. Zeelmeister and Tuzson (11) report only a 2 per cent change in 24 hours at room temperature (room temperature not specified).

The stability of the pigment toward alkali was investigated, since the pigment solution is shaken for 1 minute with potassium hydroxide. No spectroscopic change was observed when a hexane solution of pure  $\beta$ -carotene was shaken continuously with alcoholic potassium hydroxide for 1 hour. Similar treatments with oxalic and hydrochloric acids resulted in decomposition, as shown by a general 3 to 4 per cent lowering of the absorption curve.

Attempts were then made to obtain quantitative spectroscopic data for neo- $\beta$ -carotene, for use with those for  $\beta$ -carotene (15) in quantitative analysis. Purification of the pigment by crystallization did not appear promising, because  $\beta$ -carotene crystallizes out first from a mixture of the two, and because partial reversion to  $\beta$ -carotene might occur if attempts were made to concentrate an eluate of pure neo- $\beta$ -carotene from an alumina column. It has been observed previously (11, 12) that this reversion takes place on standing.

An alternative method was to make the spectroscopic observations before determination of the concentration (11, 16). A solution of the pigment, freshly eluted from the adsorption column, was studied spectroscopically, then evaporated to dryness, and the residue weighed. For this purpose a sample of pure  $\beta$ -carotene (100 mg.) was refluxed 24 hours in hexane and the neo- $\beta$ -carotene separated by means of a rapidly developed alumina column. The neo- $\beta$ -carotene was eluted and the absorption spectrum in hexane determined immediately on an aliquot of the solution. The remainder was evaporated at room temperature under reduced pressure and dried to constant weight *in vacuo* at about 15  $\mu$  of mercury. Duplicate weights agreed within 0.7 per cent. With the average of these weights, specific absorption coefficients were calculated as described later, and the curve shown in Fig. 1 was obtained. For purposes of comparison, the curve for  $\beta$ -carotene (15) is also shown. It will be noted that the specific absorption coefficient at 4360 Å. is 196, and is therefore coincident with the coefficient for  $\beta$ -carotene at this wavelength.

Another method involved a study of the absorption curve during isomerization. A value of 195 at 4360 Å. was obtained by studying the curves of solutions of  $\beta$ -carotene before and after heating at 65°. The absorption values remained constant at 4360 Å. Similar observations

have been made on solutions of  $\beta$ -carotene allowed to remain at room temperature, for periods up to 72 hours.

Table I gives the numerical values of the specific absorption coefficients of neo- $\beta$ -carotene at the maxima and minimum.

According to the method of analysis described below, the conversion

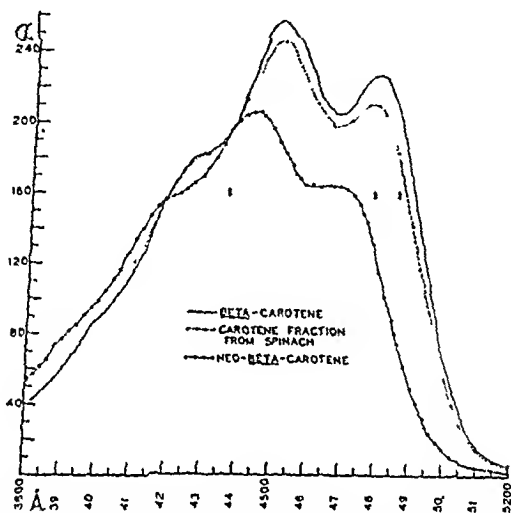


FIG. 1. Absorption spectra in hexane solution. The ordinate represents the specific absorption coefficient  $\alpha$ ; the abscissa, wave-length in  $\text{\AA}$ .

TABLE I  
Specific Absorption Coefficients for Neo- $\beta$ -carotene in Hexane Solution

Wave-length	Specific absorption coefficient
$\text{\AA}$ .	<i>l. per gm. cm.</i>
4670	166 Maximum
4625	164 Minimum
4425	206 Maximum

of  $\beta$ -carotene to neo- $\beta$ -carotene at  $30^\circ$  in the dark was studied. The results are given in Table II.

In the same way, the reversion of neo- $\beta$ -carotene to  $\beta$ -carotene was followed on a solution of neo- $\beta$ -carotene which was allowed to stand at  $40^\circ$  in the dark. The reaction was too slow at  $30^\circ$  to avoid gross decomposition. The percentages of  $\beta$ -carotene in these solutions were determined

spectrophotometrically and were qualitatively confirmed by chromatography. They were 2.6, 5.2, 18.8, and 32.3<sup>3</sup> per cent after 0, 1, 24, and 72 hours, respectively.

Table III presents typical analytical results obtained on various fresh vegetables. The reproducibility of results for the entire analytical procedure is illustrated by values for two samples from the same lot of spinach leaves which were as follows: total carotene 60.9 and 60.0  $\gamma$  per gm.;

TABLE II  
*Conversion of  $\beta$ -Carotene to Neo- $\beta$ -carotene at 30° in Hexane Solution*

Time	Neo- $\beta$ -carotene	Time	Neo- $\beta$ -carotene
<i>hrs.</i>	<i>per cent</i>	<i>hrs.</i>	<i>per cent</i>
0	0	23	14.5
0.5	1.0	32	16.5
3	2.8	45	19.5
4.5	4.5	78	21.7
9	7.5	101	25.1*

\* See foot-note 3.

TABLE III  
*Percentage  $\beta$ -Carotene in Carotene Fractions of Certain Vegetables*

Vegetable (fresh)	Total carotene per gm. fresh weight, 4360 Å.	$\beta$ -Carotene	
		4780 Å.	4850 Å.
	$\gamma$	<i>per cent</i>	<i>per cent</i>
Asparagus . . . . .	8.47	83.3	82.5
Broccoli leaves . . . . .	97.5	89.6	91.6
" tips . . . . .	29.4	82.3	83.3
" stems . . . . .	6.10	83.3	85.8
Spinach . . . . .	69.0	87.5	87.5
Peas . . . . .	4.05	86.5	87.0
Green beans . . . . .	3.40	79.2	79.1

$\beta$ -carotene 87.5 and 87.0 per cent at 4780 Å. and 86.7 and 86.2 per cent at 4850 Å. Chromatographic examination confirmed the analytical data in every instance and did not indicate the presence of significant amounts of pigments other than  $\beta$ -carotene and neo- $\beta$ -carotene in the final carotene solutions.

Samples of spinach leaves were analyzed after various heat treatments. Blanching for 30 seconds did not alter either the total carotene content

<sup>3</sup> This study was not carried to the equilibrium point because of gross decomposition after this observation.

imum of the  $\beta$ -carotene curve is here and the reference curves are also widely separated. Errors in wave-length drum calibration are less important than would be the case if the readings were taken on a steep slope.

TABLE IV  
*Analytical Constants*

Wave-length	Specific absorption coefficients in hexane solution	
	$\beta$ -Carotene	Neo- $\beta$ -carotene
$\lambda$ .	<i>l. per gm. cm.</i>	<i>l. per gm. cm.</i>
4850	200	81.3
4780	228	132
4360	196	196

TABLE V  
*Composition of Carotene Fraction from Fresh Spinach*

Wave-length	$\beta$ -Carotene	Difference between reference curves in units of $\alpha$	Wave-length	$\beta$ -Carotene	Difference between reference curves in units of $\alpha$
$\lambda$ .	<i>per cent</i>	<i>l. per gm. cm.</i>	$\lambda$ .	<i>per cent</i>	<i>l. per gm. cm.</i>
5200	81.5	3.80	4780	81.8	96.5
5150	83.8	6.15	4700	82.8	47.0
5100	85.5	12.5	4650	81.3	42.7
5050	79.6	27.0	4600	80.7	57.5
5000	83.3	47.8	4550	81.3	75.0
4950	82.5	80.0	4500	82.4	68.5
4900	82.2	106.3	4450	80.0	40.0
4850	82.3	119.0	4400	82.7	11.5
4800	81.2	111.0			
Mean.....				82.05	
Average deviation from mean.....				$\pm 1.29$	

To illustrate this calculation, the spinach carotene extract whose curve<sup>5</sup> is shown in Fig. 1 is analyzed below.

$$\log \frac{I_0}{I(4360)} = 0.341; \quad \log \frac{I_0}{I(4780)} = 0.367$$

$$C = \frac{0.341}{196} = 0.00174 \text{ gm. per liter}$$

$$\% \text{ Component 2} = \frac{\frac{0.367}{0.00174} - 132}{96} \times 100 = 82.3$$

<sup>5</sup> Significant absorption below 4100  $\text{\AA}$ . due to impurities in diacetone alcohol prevents quantitative treatment below this wave-length of the spectra of extracts in which traces of diacetone alcohol remain.

In the foregoing, Component 1 refers to neo- $\beta$ -carotene, while Component 2 refers to  $\beta$ -carotene. The above calculation for percentage composition was made for wave-lengths where the reference curves were widely separated, and a high degree of accuracy would be expected.

A test of the accuracy of the reference curves in Fig. 1 is the analysis of mixtures of the two pigments for the amount of each. Reasonably close checks were obtained at wave-lengths above 4400 Å. Table V presents the percentages of  $\beta$ -carotene in this same solution as calculated for various wave-lengths.

#### DISCUSSION

To the writers' knowledge, successful efforts to resolve the " $\beta$ -carotene" portion of a plant extract into more than one substance have not been reported. Peterson (6) has stated that "on all adsorbents the main pigment fraction adsorbs in the same position as  $\beta$ -carotene and cannot be distinguished from it when the two are adsorbed simultaneously," and that the "abnormal absorption spectra are an intrinsic property of the pigments as found in the original material previous to extraction."

Smith (18) observed spectroscopic differences between successive crystallizations of  $\beta$ -carotene and Strain has made certain observations on the spectra of carotenols which parallel those made here on  $\beta$ -carotene with respect to crystallization and heat treatment. Strain (19) observed that the spectrum of "leaf xanthophyll" was altered by crystallization (p. 47) and that solutions of luteol (p. 84), cryptoxanthol (p. 105), and zeaxanthol (p. 109) were changed by heat, resulting in decreased absorption at longer wave-lengths. In the case of zeaxanthol, he obtained the same result whether air was present or absent ((19) p. 112).

The possibility that the adsorbent is responsible for the formation of neo- $\beta$ -carotene is eliminated by the fact that the same absorption curves are obtained whether or not an adsorbent is used.

The excellent agreement in Table V for all wave-lengths between 4400 and 5000 Å. is strong evidence that the solution contained principally the two pigments under discussion. It can be noted that the average deviation from the mean of values observed at wave-lengths between 4780 and 4950 Å. is  $\pm 0.49$  per cent, which is considerably smaller than is the case for wave-lengths where the differences between reference curves are less. Thus, both spectroscopic and chromatographic evidence shows that the quantities of other pigments present in the plant material examined were negligible.

A chromatographic examination of the carotene fraction of each type of vegetable analyzed should be made on a suitable column to confirm the presence or absence of other pigments. Zechmeister and Tuzson (11) have reported that calcium hydroxide as well as alumina is a satisfactory adsorbent for this work.



The experiment on the effect of blanching, which is a common procedure prior to quick freezing of vegetables, indicates that such short exposure to heat is not likely to affect the composition of the carotene fraction seriously. The longer exposures studied caused some isomerization of  $\beta$ -carotene to neo- $\beta$ -carotene. Treatment with hot diacetone alcohol before extraction is unnecessary and may cause inaccuracy in analysis for both total carotene and  $\beta$ -carotene.

The excellent recovery of carotene added to fresh vegetables indicates that the losses of total pigment during the analytical procedure are small. The experiments with cooked vegetables indicate that enzymes do not affect the isomerization of  $\beta$ -carotene after liberation from the plant tissue as described in this method. When 3 to 4 hours are needed to complete the preparation of a group of samples, isomerization may occur to the extent of 5 per cent. Part of this isomerization may be due to the presence of plant material. The small amounts of neo- $\beta$ -carotene formed during the analysis do not account for the much larger contents of this pigment usually observed in fresh vegetables. This is strong evidence for the natural occurrence of the neo isomer.

The wide divergence of the absorption curves for  $\beta$ -carotene and neo- $\beta$ -carotene may cause any type of photometric analysis for  $\beta$ -carotene to be in error if the possible presence of neo- $\beta$ -carotene is neglected. Under certain conditions, this error may be very large, depending upon the pigment composition of the carotene fraction and the method of analysis. If, if the pigment concentration is determined spectrophotometrically, with crystalline  $\beta$ -carotene as the reference standard, the apparent concentration will vary according to the wave-length employed.

It should be emphasized that a carotene analysis tells little about the biological activity of a vegetable as a source of vitamin A unless the amount of each pigment in the carotene fraction is known, as well as the biological activity of each of these pigments. An additional factor is the relative availability of the pigments to the animal. Preliminary experiments indicate that neo- $\beta$ -carotene has significantly less vitamin A activity than  $\beta$ -carotene.<sup>6</sup>

#### SUMMARY

The natural occurrence of neo- $\beta$ -carotene in fresh plant material and the importance of this pigment in carotene determinations are indicated by the following observations.

1. Pigment fractions from fresh plant material may form homogeneous " $\beta$ -carotene" zones on a magnesia column but do not have the same absorption spectra as those of solutions from pure recrystallized  $\beta$ -carotene.

<sup>6</sup> Barrick, E. R., and Shrewsbury, C. L., unpublished results (1941).

2. The spectroscopic differences consist of relatively decreased absorption toward the red, and a slight shift of maxima toward the blue.

3. When pure  $\beta$ -carotene is heated, its absorption curve becomes similar to that of plant carotene fractions. The same type of spectrum is observed in a study of the mother liquor from recrystallized pure  $\beta$ -carotene.

4. Solutions of these three types may be chromatographically resolved into two zones on an alumina column. One component is  $\beta$ -carotene; the other is neo- $\beta$ -carotene, an isomer of  $\beta$ -carotene.

5. The quantitative absorption spectrum of neo- $\beta$ -carotene was obtained and employed as a reference standard in carotene analysis.

6. The conversion of  $\beta$ -carotene to neo- $\beta$ -carotene was studied and found to be reversible.

7. Several typical results are presented, showing the percentage of  $\beta$ -carotene in carotene fractions from various vegetables (79 to 90 per cent).

8. The loss of carotene and amount of isomerization during the analysis are small.

9. Studies on the effect of blanching and cooking indicate the absence of enzyme action on this isomerization during analysis.

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# STUDIES ON RATES OF EXCHANGE OF SUBSTANCES BETWEEN THE BLOOD AND EXTRAVASCULAR FLUID

## I. THE EXCHANGE OF WATER IN THE GUINEA PIG

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In order to evaluate data on the rate of passage of heavy water across the placenta of the pregnant guinea pig, we have found it necessary to study its behavior when injected intravenously into the adult, non-pregnant animal. Our primary effort has been to determine the changes in concentration of deuterium oxide in the blood with respect to time, and from these data to gain an estimate of the rate of exchange of the water of the blood with that of the extravascular fluid. We have also been interested in finding the total water available for dilution of deuterium oxide in the animal's body; in this respect our experiments are similar to those of Hevesy and Jacobsen (1) on the rabbit.

### *Procedure*

Five adult guinea pigs were used to determine the changes in blood concentrations of DHO with respect to time. A known quantity of 44 per cent (by weight)  $D_2O$  in isotonic solution was injected into an arm or leg vein. Blood samples, totaling no more than six per animal, of 1 to 1.5 cc. each, were taken from a cannula placed in the proximal end of a carotid artery. The water from the whole blood was secured by vacuum distillation at room temperature and condensed in tubes surrounded by solid  $CO_2$ . The purification of the water and the measurement of the concentration of heavy water were carried out by the technique described by Keston, Rittenberg, and Schoenheimer (2). This method involves timing the fall of a drop of water of fixed volume through an immiscible liquid with a density slightly lower than that of water.

Preliminary experiments in which the dye T-1824 was injected intravenously into four adult guinea pigs were used to estimate the plasma volume of the guinea pigs and the rate of mixing of the injected  $D_2O$ . The technique of estimation of T-1824 with the Evelyn photoelectric colorimeter was as described by Gibson and Evelyn (3). Our determina-

\* Paper No. 221.

tions have been made on samples with a depth of 1 mm. and with a dye concentration of 0.02 to 0.03 mg. per cc.

### RESULTS AND DISCUSSION

The plan of the experiments has been to follow variations in the concentration of intravenously injected DHO in the blood with respect to time. The rate of loss of DHO is to be used to evaluate the rate of flow of water from blood to extravascular fluid. There is ample evidence that DHO in the concentration used here behaves equivalently to  $H_2O$  (4, 5).

In order to measure the rate of disappearance of DHO from the blood, by analyzing samples from the carotid artery, it is of course necessary that the DHO be thoroughly mixed with the water of the blood. Our observations on T-1824 indicate that mixing is at least 95 per cent complete at the end of 1 minute after injection, and agree with those of Went and Drinker (6) that it is complete after 3 minutes. The passage of a substance back and forth between the vascular and extravascular fluids is in itself a mixing process and, as will be shown subsequently, this is exceedingly rapid for heavy water. It was therefore concluded that measurements taken after 1 minute subsequent to injection gave valid evidence on concentration, but the single measurement in our series taken less than 1 minute after injection was excluded from the determination of the rate of transfer of DHO from blood to extravascular fluid.

The observations on concentration of DHO in the water of the blood are given in Table I. In order to place the measurements for the different animals on a common basis, it is necessary to adjust them to the same initial concentration. To do this the volume of the water in the blood relative to the total body weight was estimated. From the experiments with T-1824 mentioned above, it was found that the average plasma volume was 4.3 cc. per 100 gm. of body weight; this value is greater than that found by Went and Drinker (6), using a micromethod with vital red on a series of guinea pigs weighing considerably less than ours. Smith, Arnold, and Whipple (7) found the plasma volume to be 52.2 per cent of the total blood volume in the adult dog. This proportion was assumed to hold for the adult guinea pig, which leads to a blood volume value of 8.3 cc. per 100 gm. of body weight, or a blood weight of 8.8 per cent of the body weight (sp. gr. 1.06). We have dried guinea pig blood and found that 85 per cent of the weight of the blood is water, which leads finally to the estimate that there are on the average 7.5 cc. (or 7.5 gm.) of water in the blood per 100 gm. of body weight. With this estimated volume of the water of the blood and the known amount of  $D_2O$  injected (Table I), the initial concentration of DHO in the blood can be calculated for each ex-

periment on the assumption of uniform distribution. These initial concentrations varied from 0.914 to 2.35 gm. of DHO per 100 cc. of water in the blood; to place the observations on a common basis they were all adjusted to an initial concentration of 1 gm. per 100 cc.

The change of concentration of DHO in the water of the blood, with respect to time, is shown in Fig. 1. In order to derive the rate of movement of water from these data, it is assumed that the amount of DHO lost from the blood per unit of time is proportionate to (1) the number of cc. of water which move from blood to extravascular fluid per unit of time and (2) the amount of DHO present in each cc. of this water. Part of the DHO which escapes into the extravascular fluid will return to the blood

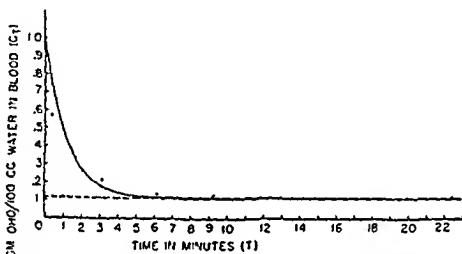


FIG. 1. Change in concentration of DHO in the water of the blood with respect to time. The points were derived by adjusting the observations of Table I to a  $c_i$  value of 1 gm. of DHO per 100 cc. of water in blood, as explained in the text. The equation for the curve is  $c_t - 0.115 = 0.885e^{-\frac{0.73}{0.885}t}$  and has been derived from the observations at 1.3 minutes and beyond. The equilibrium value was constant up to 2 hours.

and the amount which returns per unit of time is proportionate to (1) the rate of movement of water from extravascular fluid to blood and (2) the concentration of DHO in the extravascular fluid. From these considerations it is evident that

$$(1) \quad \frac{dh_t}{dt} = -r \frac{h_t}{w_b} + r \frac{(h_0 - h_t)}{w_f}$$

where

$r$  = volume of water passing from blood to extravascular fluid (or from extravascular fluid to blood) per unit of time

$h_0$  = number of units of DHO in the blood at zero time

$h_t$  = number of units of DHO in the blood at any subsequent time  $t$

$w_b$  = total volume of water of the blood

$w_f$  = volume of extravascular water available for dilution of DHO

The rate of change in concentration of DHO in the water of the blood,  $c_t$ , is obtained by dividing Equation 1 by  $w_b$ .

$$(2) \quad \frac{dc_t}{dt} = -\frac{r}{w_b} c_t + \frac{r}{w_f} (c_0 - c_t)$$

Integrating Equation 2 and solving for the constant of integration gives

$$(3) \quad g[\ln(c_t - c_{eq.}) - \ln(c_0 - c_{eq.})] = -Rt$$

where

$R = r/w_b$  = proportion of total water in the blood which passes into the extravascular fluid per unit of time

$g = w_f/(w_b + w_f)$  = proportion of the total available water which is extravascular

$c_{eq.}$  = concentration of heavy water in the water of the body at equilibrium

Equation 3 states that the concentration of DHO in the water of the blood approaches equilibrium in such a way that the logarithm of the concentration in excess of the equilibrium concentration is a linear function with time.

In exponential form Equation 3 may be expressed

$$(4) \quad c_t - c_{eq.} = (c_0 - c_{eq.}) e^{-\frac{R}{g}t}$$

which states that the excess concentration is reduced by a constant proportion per unit of time.

In Equations 3 and 4,  $c_0$  is 1 gm. per 100 cc., since the concentrations for all experiments were adjusted to this basis. All of the other constants of the equations except  $R$  are to be derived directly from the data of Table I and the results on determination of plasma volume with the dye T-1824. These constants are derived as follows:

$w_b$  stated per 100 gm. of body weight = 7.5 cc., as previously discussed.

$w_f$  stated per 100 gm. of body weight is obtained by determining the total water of the body available for dilution of DHO and subtracting from this  $w_b$ . The total water available for dilution of DHO was determined for each animal from the known quantity of  $D_2O$  injected and the final concentration of DHO in the water of the blood. Expressed in ratio to the body weight, it varied from 60 to 67 cc. per 100 gm. of body weight and averaged 65 cc. per 100 gm. of body weight. This value agrees well with that of McDougall *et al.* (8) who used  $D_2O$  in the rat and with that of Painter (9) who used urea and sulfanilamide in the dog. These values are considerably lower than those reported by Hevesy and Jacobsen (1) for the rabbit.

$g = w_f/(w_b + w_f)$ . Since the water of the blood has been found to average 7.5 cc. per 100 gm. of body weight, and the total water available

for dilution of DHO to average 65 cc. per 100 gm. of body weight,  $q = (65 - 7.5)/65 = 0.885$ .

$c_{eq.} = h_0/(w_b + w_f) = c_0[w_b/(w_b + w_f)] = 0.115$  gm. of DHO per 100 cc. of water on the basis of an initial concentration of DHO equal to 1 gm. per 100 cc.

The evaluation of these constants now makes it possible to plot the logarithmic function of  $c_t$  given by Equation 3 against  $t$ . When this is done, it is found that the points fall about a straight line the slope of which

TABLE I

*Change of Concentration of DHO in Water of Blood, with Time*

These data are necessary for evaluation of the rate of exchange of water between blood and extravascular fluid. The concentration of  $D_2O$  injected was 44 per cent but for convenience the amount injected is expressed in terms of 100 per cent  $D_2O$ .

Experiment No.	Amount of 100 per cent $D_2O$ injected	Weight of animal	Time after injection	Concentration of DHO in water of blood
	gm.	gm.	min.	gm. per 100 cc.
1	0.485	702	6.2	0.123
			20.0	0.106
2	0.485	685	19.6	0.107
3	0.538	785	3.1	0.164
			9.6	0.120
			17.2	0.102
4	0.653	861	0.37	0.577
			1.7	0.341
			3.1	0.256
			22.5	0.127
5	1.43	812	1.3	0.686
			2.1	0.624
			3.2	0.500
			13.6	0.271

is equal to  $R$  (values of  $c_t$  near equilibrium are to be excluded in this treatment of the data, because small numerical fluctuations in these values appear as large fluctuations in the values of the logarithms).

For the sake of greater accuracy in the determination of  $R$ , however, Equation 4 was fitted by the method of least squares<sup>1</sup> to the observations of Fig. 1 from 1.2 to 9.6 minutes inclusive. The value at  $t = 0.37$  was omitted because of incomplete mixing, and the values beyond  $t = 9.6$  had already been used to determine the equilibrium value. The equation thus

<sup>1</sup> The deviations used were  $c_t$  deviations weighted by the factor  $(c_0 - c_t)/(c_0 - c_{eq.})$ .



derived is plotted against the observations in Fig. 1 and appears to give a reasonably good description of the change of concentration with respect to time. The value of  $R$ , obtained from the fitted equation, with  $t$  expressed in minutes, is 0.73 with a probable error of  $\pm 0.05$ . This states that a volume of water equal to 73 per cent of the volume of the water of the blood flows from the blood vessels into the extravascular fluid per minute. This in terms of total blood volume means that in the guinea pig a volume of water equal to 66 per cent of the blood volume is exchanged between blood vessels and extravascular fluid each minute.

We are much indebted to Dr. David Rittenberg for considerable advice about the apparatus and for checking several of our results. We are also grateful to Dr. Leslie Hellerman and Dr. Edgar J. Poth for aid in the interpretation of the data.

#### SUMMARY

1. When deuterium oxide is used, the evidence indicates that in the guinea pig 73 per cent of the water in the blood is exchanged with extravascular water every minute. The probable error on this rate is 5 per cent.

2. Deuterium oxide, injected intravenously, is diluted at equilibrium by a volume of water amounting to 65 cc. per 100 gm. of body weight.

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## STUDIES IN AMINO ACID METABOLISM

### VIII. THE METABOLISM OF *l*(-)-HISTIDINE IN THE NORMAL RAT\*

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The transformation of amino acids to sugar has been quite fully investigated by means of phlorhizinized animals (1). Although this technique has given some very valuable information, the fact remains that the experimenter is dealing with an animal which is in an unphysiological condition. In some instances an entirely different picture is given when the amino acids are fed to normal animals. The results are not only quantitatively but also qualitatively different. Probably the most striking discrepancy is in the case of *dl*-phenylalanine and *l*(-)-tyrosine. Experiments on phlorhizinized animals (1) indicate that these two aromatic amino acids are precursors of the acetone bodies. On the other hand, definite glycogen formation followed the feeding of these compounds to normal rats (2, 3). A decrease in an experimentally produced acetonuria also resulted after these two acids were fed (2).<sup>1</sup>

One of the hexone bases, *l*(+)-arginine, has been considered as an amino acid capable of giving rise to a relatively large amount of sugar (1). Unquestionably this is true when the phlorhizin technique is employed, but when this acid is fed to normal rats the evidence indicates that the amount of glycogen formed is quite small (4). Under similar experimental conditions a second hexone base, *dl*-lysine, failed to show any sugar or acetone body formation (4).

Dakin (1) was unable to obtain any clear cut evidence of "extra sugar" production when amounts of histidine ranging from 8.38 to 13.2 gm. were injected into phlorhizinized dogs.

The present study was undertaken to furnish some information as to the rôle of *l*(-)-histidine in carbohydrate metabolism.

#### EXPERIMENTAL

Female rats ranging in weight from 100 to 180 gm. were placed for 9 days on a diet high in fat and carbohydrate and low in protein, as described

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<sup>1</sup> Butts, J. S., unpublished experiments.

by Deuel *et al.* (5). This is the minimum time for the development of a maximum ketonuria when the animals are allowed to fast (5).

At the end of the 9 day period the animals were subjected to a 24 or 48 hour fast and then confined to separate metabolism cages placed on large glass funnels which permitted the collection of 24 hour samples of urine. The urine was analyzed for total acetone bodies according to the Van Slyke procedure and total nitrogen by the Kjeldahl method. In Experiment I, total imidazoles were determined colorimetrically, with a Klett-Summerson photoelectric colorimeter equipped with a No. 540 filter (6).

TABLE I

*Total Acetone Body Excretion from Female Rats after 9 Days on High Fat Diet*

In Experiment I the animals were fasted 24 hours before receiving the *l*(-)-histidine. In Experiment II they were fasted 48 hours before receiving the amino acid. Group 1 in both experiments received 0.45 mm per 100 sq. cm. of *l*(-)-histidine per day, while Group 2 received twice this amount. Group 3 received 4 cc. of 7.5 per cent sodium chloride solution per day. No other food was given during the days listed.

The days are numbered from the beginning of the preliminary fasting period.

Experiment and group No.	Total acetone body and nitrogen excretion, mg. per 100 sq. cm.							
	2nd day		3rd day		4th day		5th day	
	Acetone	Nitrogen	Acetone	Nitrogen	Acetone	Nitrogen	Acetone	Nitrogen
I-1	28.5 (6)	42.0 (6)	20.4 (3)	44.9 (3)	12.4 (3)	37.9 (3)		
2	31.8 (6)	55.6 (6)	12.6 (4)	61.9 (4)	2.8 (3)	59.6 (3)		
3	17.1 (6)	31.3 (6)	40.1 (6)	29.4 (6)	33.8 (6)	30.3 (6)		
II-1	14.7 (8)*		21.8 (8)	47.3 (8)	4.1 (8)	48.4 (8)	3.2 (8)	52.1 (8)
2	10.3 (6)		23.3 (5)	61.0 (5)	2.7 (4)	66.7 (4)	2.8 (3)	60.9 (3)
3	7.5 (4)		36.3 (4)	27.5 (4)	32.8 (4)	25.4 (4)	22.8 (4)	26.3 (4)

The numbers in parentheses indicate the number of experiments in each group.

\* No histidine fed in Experiment II on this day.

Since such a small amount of the material was found in the urine, these determinations were not extended to any of the other experiments.

The histidine monohydrochloride used in the experiments reported in this paper was a commercial product of high purity. The amino acid was dissolved in water and the pH brought to 6.5. The final concentration was adjusted so that 42.9 mm of histidine were contained in 100 cc. of the solution.

Eighteen female rats were used in Experiment I. At the end of the 24 hour fast period the animals were weighed and the surface area of each was calculated according to Lee's formula (7). Group 1, composed of six animals, received 0.45 mm of *l*(-)-histidine per 100 sq. cm. per day, fed in two doses. Group 2 received twice this amount. Group 3 received 4 cc.

of a 7.5 per cent sodium chloride solution each day. All feedings were made by stomach tube.

In Experiment II the fasting period was increased to 48 hours. Urine collections were made during the 2nd fast day (Table I, 2nd day) but no amino acid was administered. This experiment extended through a 5 day period.

The experiments on liver glycogen formation were begun after a 48 hour fast. An attempt was made to determine liver glycogen after allowing maximum absorption, as had been done in earlier studies on other amino acids (8, 9). It was found that when more than 4.40 mm of the amino acid were fed toxic symptoms resulted. Because of this an amount of histidine averaging 2.58 mm was fed during the first 3 hours of the experiment. No further feedings of the amino acid were made, with the exception of two animals in the 8 hour group which received 4.30 mm. The time elapsing between feeding the first dose and sacrificing the animal ranged from 4 to 20 hours. The method of Good, Kramer, and Somogyi (10) was used for the glycogen determination.

### Results

In Table I (Experiment I) the results of feeding the *l*(-)-histidine to animals after 9 days on the high fat diet are recorded. The 1st experimental day is omitted, as this was a fasting period designed to lower liver glycogen. No urine collections were made. It is evident that the animals fed the amino acid showed a definite progressive decrease in the acetonuria, and that the larger dose of *l*(-)-histidine caused a greater effect than the smaller dose. In 50 per cent of the experiments in which the amino acid was fed, toxic symptoms resulted as manifested by a severe hematuria. This seemed to occur almost exclusively in the smaller animals in each group. No results of experiments in which any toxicity was evident are reported.

In a second series of experiments recorded in Table I (Experiment II) exactly the same picture is given. The 2nd day shows the level of the ketonuria when no amino acid was fed. In the control groups during the next 2 days the level of acetone body excretion was fairly constant, followed by a drop on the 5th day. The animals receiving the amino acid showed an immediate decrease in the acetonuria which continued throughout the experiment.

In all of the experiments recorded in Table I the nitrogen excretion is included. A significant increase occurred after the small dose of histidine was fed, while the larger amount of the amino acid caused a more marked increase in excretion.

The urinary excretion of total imidazoles after *l*(-)-histidine was fed is reported in Table II. It is evident that the rat can tolerate large amounts of *l*(-)-histidine.

The results of the glycogen studies are reported in Table III. No glycogen formation occurred until the 12 hour period, with a maximum formation at 16 hours. By 20 hours the level had begun to decrease.

TABLE II

*Excretion of Total Imidazoles by Female Rats after l(-)-Histidine Feeding*

For detailed explanation of the groups, see Table I, Experiment I.

Group No.	Urinary imidazoles (calculated as histidine), as per cent of histidine fed		
	2nd day	3rd day	4th day
	per cent	per cent	per cent
1	2.7 (6)	3.1 (4)	4.6 (3)
2	4.9 (6)	3.0 (4)	6.1 (4)
3	0.0 (6)	0.0 (6)	0.0 (6)

The numbers in parentheses indicate the number of animals in each group.

TABLE III

*Effect of l(-)-Histidine on Liver Glycogen*

Female rats were fed 0.98 mm of *l*(-)-histidine per 100 sq. cm. of body surface and sacrificed at various time intervals. Control animals received 7.5 per cent sodium chloride solution. The total amount was fed during the first 3 hours of the experiment.

	4 hrs.		8 hrs.		12 hrs.		16 hrs.		20 hrs.	
	Glyco- gen per cent	S.D.M.	Glyco- gen per cent	S.D.M.	Glyco- gen per cent	S.D.M.	Glyco- gen per cent	S.D.M.	Glyco- gen per cent	S.D.M.
<i>l</i> (-)-Histidine	0.24 (6)	0.09	0.16* (8)	0.03	0.41 (6)	0.03	1.08 (6)	0.08	0.39 (6)	0.11
Control	0.13 (4)	0.03	0.14 (6)	0.05	0.19 (4)	0.08	0.24 (6)	0.03	0.16 (4)	0.01

The numbers in parentheses refer to the number of animals in each group. The animals in each group averaged 160 gm. in body weight.

\* Two animals in this group received 1.63 mm per 100 sq. cm.

#### DISCUSSION

It is rather surprising that after *l*(-)-histidine has been fed more than 8 hours must elapse before there is any indication that glycogen formation is occurring. As Table III shows, this deposition is at a maximum during the 16 hour period. Thus, histidine may be similar to glycine in this respect, as MacKay *et al.* (11) have reported that no appreciable glycogen

deposition follows the feeding of this amino acid until more than 8 hours have elapsed.

The most likely explanation of this unusual behavior would be very slow absorption, although Doty and Eaton (12) have reported that histidine is absorbed quite rapidly. On the basis of mg. per 100 gm. of rat per hour the rate of absorption of histidine hydrochloride was 97.4 for the 1st hour and 71.0 for the 2nd hour. In our experiments, which we are not reporting in detail but in which the technique was comparable to that used by Doty and Eaton, we found 51.4 mg. per 100 gm. of rat per hour for the 4 hour period as an average of six experiments and 39.8 mg. for two experiments for the 8 hour group. This would indicate that absorption is not a controlling factor, since the amount of histidine fed was on an average 2.58 mm and this amount would have been almost completely absorbed in 6 to 8 hours, with by far the greatest amount absorbed during the first 3 to 4 hours.

It is of interest that the experiments in which *l*(-)-histidine was fed to rats with a ketonuria support the conclusion that a latent period exists before noticeable carbohydrate formation occurs. In each group of animals (Table I, Experiments I and II) on the 1st day that the histidine was fed there was little effect on the acetonuria. In all experiments on the succeeding days, however, the amino acid caused a significant lowering of the acetone body output.

The argument is often advanced that glycogen formation after an amino acid is fed is a secondary effect and is not caused directly by the amino acid. The fact that the decrease in the ketonuria seems to parallel the glycogen formation as far as the latent period is concerned might be interpreted as evidence that the amino acid is the precursor of the glycogen.

The nitrogen excretion shows the expected increase in the animals fed the amino acid. Although the urea output is not known, the results on histidine excretion establish the fact that no appreciable amount of the amino acid is being excreted.

#### SUMMARY

1. Liver glycogen formation follows the feeding of *l*(-)-histidine, with the maximum deposition occurring at 16 hours.

2. A period of more than 8 hours occurs before there is evidence of glycogen formation following the feeding of the amino acid.

3. In many cases a severe hematuria develops after *l*(-)-histidine is fed to rats.

4. A ketonuria produced by a high fat diet is decreased by feeding *l*(-)-histidine.

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# THE EFFECT OF ADDED CYSTINE IN PURIFIED RATIONS FOR THE CHICK\*

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Griffith (1) showed that hemorrhagic degeneration in rats was aggravated by the addition of cystine to a low fat, low choline diet containing 15 per cent of casein. We attempted, therefore, to determine the effect of cystine supplementation to various purified rations in our work with chicks. Although supplementary cystine produced no effect in low choline rations, it was found that the addition of cystine to certain rations containing 18 per cent of casein and sufficient choline (0.15 per cent) improved the rate of growth. Since these basal rations were similar to those which we were using in studies on the "cartilage growth factor" (2-4), an attempt was made to correlate this work with the chick's requirement for cystine. This paper deals with the effect of added cystine in purified rations containing 18 per cent of casein.

## EXPERIMENTAL

The experimental procedure and the method for determining total creatinine (modified for the Evelyn photoelectric colorimeter) have been described (5). Day-old white Leghorn chicks were divided into groups of six and placed on experiment for 4 weeks. The basal ration (No. 478) had the following composition per 100 gm.: dextrin 67, casein (alcohol-extracted) 18, Salts 4 (6) 5, dicalcium phosphate 1, soy bean oil 5, kidney residue<sup>1</sup> 2, solubilized liver extract 2, thiamine 300  $\gamma$ , pyridoxine 400  $\gamma$ , riboflavin 400  $\gamma$ , pantothenic acid 1.5 mg., choline 150 mg., inositol 100 mg., and nicotinic acid 10 mg. 2 drops of haliver oil, fortified with vitamin

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<sup>1</sup> The residue after exhaustive extraction of defatted kidney with water, 50 per cent ethanol, and 50 per cent methanol.



D, were fed by dropper weekly. Supplements were fed at the expense of dextrin. The casein was prepared as follows: crude casein was washed repeatedly with large volumes of dilute hydrochloric acid, dissolved in dilute ammonium hydroxide, precipitated with hydrochloric acid, and dried. The kidney residue was fed as a source of biotin, and the solubilized liver extract supplied the *Lactobacillus casei* factor (7). Commercially obtained *l*-cystine, *d*-arginine monohydrochloride, and glycine were used throughout.

### Results

Growth results will be considered first (Table I). Arginine and glycine, together, promoted growth as already reported (4, 8). The addition of 0.2 per cent of cystine with the arginine and glycine caused a marked increase in the rate of growth, although maximum growth was not obtained until at least 0.3 per cent of cystine was fed. Levels of cystine higher than 0.5 per cent proved somewhat detrimental to growth. When 0.5 per cent of methionine was fed in place of cystine, good growth was also obtained (Experiment 7). Cystine, when fed without arginine and glycine supplementation to the basal ration, was ineffective. Results obtained when various levels of cystine were fed with gelatin were similar to those obtained with arginine and glycine except that 0.2 per cent of cystine gave maximum growth. Since gelatin, fed as a source of arginine and glycine, contains 0.8 to 0.9 per cent of methionine (9, 10), it supplies nearly 0.1 per cent of methionine to the ration. This probably accounts for the difference in growth between the groups receiving gelatin and those receiving arginine and glycine, as well as for the difference in the amount of cystine needed for maximum growth.

When fed with arginine, glycine, and chondroitin (Experiments 14 to 16), cystine likewise improved the rate of growth. Evidence that cystine (or its equivalent of methionine) is supplied by cartilage was shown by feeding cystine in addition to the cartilage. No increase in the rate of growth was noted.

In one group a biotin concentrate<sup>2</sup> was successfully substituted for kidney residue (Experiment 19). The sole "crude" product in the ration, then, was 2 per cent of solubilized liver extract, which was evidence that all the unknown factors necessary for growth were supplied by this fraction. Besides that contained in the liver extract, the entire protein requirement of the chick was met by 18 per cent of casein, 10 per cent of gelatin (for arginine and glycine), and 0.3 per cent of cystine. The weight of this group compared favorably to the weight of several control groups receiving

<sup>2</sup> S. M. A. No. 5000.

a practical ration, which grew "normally" under our experimental conditions.

Creatinine analyses were made on many of the groups. The figures given in Table I are the averages of the analyses of the thigh muscles of individual chicks. It was found that the total creatinine was lowered by

TABLE I  
*Results Obtained by Feeding Cystine and Other Supplements to Chicks on a Ration Containing 18 Per Cent of Casein*

Experiment No.	Supplement to Ration 478	No. of groups (6 chicks per group)	Average weight at 4 wks.	Total creatinine of fresh muscle	Gizzard lining value*
			gm.	mg. per gm.	
1	None	4	75	3.37	29
2	0.5% arginine + 3% glycine	4	126	3.98	38
3	0.5% " + 3% " + 0.2% cystine	1	198	3.45	46
4	0.5% " + 3% " + 0.5% "	2	231	3.24	45
5	0.5% " + 3% " + 1.0% "	3	167	3.19	61
6	1.0% " + 3% " + 0.3% "	1	229	3.76	62
7	0.5% " + 3% " + 0.5% methionine	1	212	3.34	50
8	0.5% cystine	1	86	3.02	21
9	10% gelatin (Knox)	4	179	3.94	52
10	10% " " + 0.2% cystine	4	239	3.75	46
11	10% " " + 0.3% "	2	235	3.69	58
12	10% " " + 0.5% "	5	233	3.79	67
13	10% " " + 1% "	3	220	3.73	71
14	0.5% arginine + 3% glycine + 5% chondroitin	2	139	4.01	56
15	As in Experiment 14 + 0.5% cystine	1	223	3.34	79
16	" " " 14 + 1% "	2	177	3.03	83
17	10-15% cartilage	11	226	4.01	68
18	10% cartilage + 0.5% cystine	2	216	3.53	73
19	As in Experiment 11 (biotin in place of kidney residue)	1	223		
20	Practical ration	2	226	3.89	96

\* The gizzard linings have been scored on the following basis: 0 = severe erosion, 25 = marked erosion, 50 = slight erosion, 75 = very slight erosion, 100 = no erosion.

cystine when fed in addition to arginine and glycine, with or without chondroitin. This may be explained on the basis that the increased metabolism due to the cystine intake has lowered the amount of arginine available for creatine formation (5, 8, 11), because the marked fall in creatinine was not noted in these groups receiving gelatin, which nearly doubles the arginine content of the ration when fed at 10 per cent. Further

2. Cystine when fed with arginine and glycine aids in the prevention of gizzard erosion. The action of these amino acids (especially cystine) explains, in part, the anti-gizzard erosion properties of cartilage. Chondroitin likewise helped to prevent gizzard erosion but showed no growth activity in our experiments.

3. The combination of arginine, glycine, chondroitin, and cystine (or its equivalent of methionine) constitutes the original "cartilage growth factor" and may be substituted for cartilage with similar results as to growth and the prevention of gizzard erosion.

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## GLUTATHIONE

### II. THE METABOLISM OF GLUTATHIONE STUDIED WITH ISOTOPIC AMMONIA AND GLUTAMIC ACID\*

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It has been shown in a recent investigation (1) that glycine (labeled with  $N^{15}$ ), when administered to rats or rabbits, is much more rapidly incorporated into glutathione (GSH) of liver and intestine than into the proteins of the same tissues. In the liver GSH of rabbits killed  $2\frac{1}{2}$  hours after the administration of the labeled glycine, two-thirds of the total isotope present was in the glycine, about one-fifth in the glutamic acid.

We have now investigated the utilization of ammonia and of *dl*-glutamic acid for GSH synthesis. Our findings furnish further evidence for the great metabolic activity of GSH.

#### EXPERIMENTAL

Isotopic ammonia as ammonium citrate was administered by stomach tube to two rabbits from which food had been withheld for 17 hours. Rabbit A received 108 mg. of nitrogen which contained 4.51 atom per cent excess  $N^{15}$ , Rabbit B 30.9 mg. with an isotope concentration of 29.0 atom per cent excess. Both animals were killed  $2\frac{1}{2}$  hours after the administration of the isotopic ammonia. The isolation of GSCu and the other nitrogenous fractions (Table I) was carried out as previously described (1). Glutamic acid was isolated from the protein by means of the Foreman procedure (2). The cysteine fraction of GSH and protein was isolated according to Graff, Maculla, and Graff (3) (see (1) foot-note 5). The amide nitrogen from the protein hydrolysates and the ammonia from urine were obtained by alkalizing with baryta and aerating the ammonia for 4 hours into sulfuric acid. All nitrogenous samples were analyzed for their isotope concentrations (Table I). For the isolation of urea the non-protein fraction which remained after the precipitation of GSH as the cadmium compound was brought to dryness and the residue was extracted with boiling ethanol (95 per cent). The alcoholic solution was brought to dryness and the residue was taken up in water (15 ml.). To 5 ml. of this solution 10

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TABLE I

*N<sup>15</sup> Concentration of Nitrogenous Constituents of Liver, Intestine, and Urine of Rabbits Which Had Received Isotopic Ammonia*

Rabbit A, 108 mg. of N as ammonium citrate (4.51 atom per cent excess); Rabbit B, 30.9 mg. of N as ammonium citrate (29.0 atom per cent excess).

Nitrogenous constituent	Atom per cent excess found in				
	Liver		Intestine		Urine
	Rabbit A	Rabbit B	Rabbit A	Rabbit B	Rabbit B
Glutathione*	0.102	0.217	0.052	0.064	
GSH glycine†		0.163			
" cysteine		0.041			
(" glutamic acid)‡		(0.447)			
Non-protein nitrogen	0.146	0.176§	0.141	0.185	
Urea		1.18			
Protein					
Nitrogen	0.015	0.020	0.008	0.011	
Amide N	0.023	0.032	0.019	0.050	
Glutamic acid¶	0.032	0.048	0.021	0.039	
Cystine		0.009		0.010	
Nitrogen					0.207
Ammonia					0.041
Urea					0.216

\* Liver, Rabbit A, GSCu 72 mg., N 11.1 per cent; Rabbit B, GSCu 106 mg., N 11.35 per cent. Intestine, Rabbit A, GSCu 30 mg., N 11.2 per cent; Rabbit B, GSCu 22 mg., N 11.1 per cent.

† Toluenesulfonylglycine m.p. = 147° uncorrected.

‡ The glutamic acid of this sample was lost. The isotope concentration was calculated from the known values of the GSH, glycine, and cysteine fraction.

§ N 101 mg.

|| Urea N 4.7 mg.

¶ Glutamic acid hydrochloride, liver, Rabbit A, N 7.7 per cent; Rabbit B, N 7.8 per cent. Intestine, Rabbit A, N 7.6 per cent; Rabbit B, N 7.7 per cent.

TABLE II

*N<sup>15</sup> Concentration in Nitrogen of Nitrogenous Fractions of Rats Given dl-Glutamic Acid (1.10 Atom Per Cent Excess N<sup>15</sup>)*

Nitrogenous fraction	Liver		Intestine (small)	
	N <sup>15</sup> found in fraction	Nitrogen derived from administered glutamic acid*	N <sup>15</sup> found in fraction	Nitrogen derived from administered glutamic acid*
	atom per cent excess	per cent	atom per cent excess	per cent
Glutathione†	0.023	2.07	0.041	3.64
Non-protein‡ nitrogen	0.022	2.0	0.091	8.2
Protein glutamic acid§	0.010	0.9		

\* The values are calculated for an N<sup>15</sup> concentration of 100 atom per cent in the glutamic acid administered.

† Liver, 22 mg. of GSCu, N 11.0 per cent; intestine, 3 mg. of GSCu, N 11.4 per cent.

‡ Liver, 49.1 mg.; intestine, 33.2 mg.

§ N 7.7 per cent.

ml. of glacial acetic acid were added, the solution was filtered, and finally 6 ml. of a 10 per cent solution of xanthidrol in methanol were added. After 6 hours of shaking, the precipitate was filtered on a sintered glass filter and washed thoroughly with 5 per cent acetic acid in methanol and dried at 105° (m.p., 254° uncorrected). The urea of the urine was precipitated directly with xanthidrol and washed as described above (m.p., 257° uncorrected).

In another experiment 183 mg. of *dl*-glutamic acid hydrochloride containing 1.10 atom per cent excess  $N^{15}$  (4) were fed to each of three rats by stomach tube. The animals were killed after 2 hours. The GSH and non-protein nitrogen of the liver and intestine and the glutamic acid of the liver protein were analyzed for their isotope concentration (Table II).

#### DISCUSSION

*Ammonia*—In the experiments in which isotopic ammonia was fed to rabbits, the absolute amount of ammonia administered and the isotope concentration of the samples were different, but the results are expressed on a comparable basis by calculating the percentage of nitrogen derived from the administered sample (Table III).

It has been shown (5) in experiments of 5 and 9 days duration that immature and adult rats are able to utilize ammonia for amino acid synthesis. In this experiment the GSH isolated from the liver and intestine of Rabbits A and B contained appreciable amounts of isotope. About 69 per cent of the total GSH nitrogen which had been derived from the administered ammonia was present in the glutamic acid. Since the amino group of the glutamic acid of GSH is free, two mechanisms might account for its introduction: (a) formation of GSH from glutamic acid synthesized elsewhere or (b) replacement of the amino group of GSH glutamic acid still in peptide linkage. The GSH glycine contained about 4 times as much isotope as the cysteine. This may result either from a more rapid synthesis of glycine or from its more rapid incorporation into GSH or both.

The glutamic acid of the liver protein (Rabbit B) contained one-tenth of the isotope concentration of the GSH glutamic acid. This result furnishes further evidence that the metabolism of GSH is much more rapid than that of the proteins of the same organs.

The isotope concentration of the amide nitrogen of the proteins is higher than that of the protein nitrogen; for the intestinal proteins it exceeds that of the glutamic acid.

Calculated in absolute amounts, the proteins of the liver contained about twice as much isotope as the non-protein fraction, indicating the ready utilization of the administered ammonia for protein synthesis. As in similar experiments with rats (5), the portion of nitrogen derived from the

administered ammonia is higher in the urea than in the ammonia of the urine.

It can be computed from the data in Table I that there were present in the non-protein nitrogen 6.3 micromoles excess of  $N^{15}$  and that urea isolated accounted for 2.0 micromoles. Since no effort was made to isolate all of the urea but only a pure specimen, this amount (32 per cent) represents a minimum value.

It has been shown (1) in our previous experiments with rabbits that half of the glycine nitrogen of liver GSH is replaced by dietary glycine nitrogen

TABLE III

*Per Cent of Nitrogen of Various Fractions Derived from Administered Ammonia*

The values are calculated for an  $N^{15}$  concentration of 100 atom per cent in the ammonia administered.

Nitrogenous constituent	Liver		Intestine		Urine
	Rabbit A	Rabbit B	Rabbit A	Rabbit B	Rabbit B
Glutathione.....	2.26	0.75	1.15	0.22	
GSH glycine.....		0.56			
" cysteine.....		0.14			
" glutamic acid.....		1.55			
Non-protein nitrogen.....	3.24	0.61	3.13	0.64	
Urea.....		4.07			
Protein					
Nitrogen.....	0.33	0.07	0.18	0.04	
Amide nitrogen.....	0.51	0.11	0.42	0.17	
Glutamic acid.....	0.71	0.17	0.47	0.13	
Cystine.....		0.031		0.034	
Nitrogen.....					0.71
Urea.....					0.74
Ammonia.....					0.14

in less than 18 hours. We arrived at this value by assuming that the glycine introduced into GSH was not diluted by body glycine. The same calculation for the liver GSH of the rat gave a corresponding value of 8 hours. Both of these values are maximal, since the dietary glycine is indubitably diluted by non-isotopic glycine. As pointed out (1), the isotope concentration found in hippuric acid after feeding benzoic acid and labeled glycine to rats might be representative of the glycine dilution in the non-protein nitrogen fraction. Since 50 per cent of the glycine of hippuric acid originated in the dietary glycine, such a dilution of the dietary glycine would reduce to about 4 hours the period within which half of the GSH glycine of the rat liver was replaced.

The urea of the liver (Rabbit B) contained an isotope concentration 2.6 times higher than the GSH glutamic acid. The isotope concentration of the urea of the liver might be considered as approximately representative of that in the ammonia available to the cells for synthesis of other nitrogenous compounds. Since urea is synthesized at a very rapid rate, it is probably only slightly diluted by urea synthesized before the administration of the isotopic ammonia. If the isotope concentration of the liver urea corresponds to that of the ammonia from which the GSH glutamic acid was synthesized, more than one-third of the glutamic acid would have been formed and incorporated into the liver GSH of the rabbit within the experimental period of 2.5 hours. This value is only a crude approximation, since we do not know to what extent the glutamic acid may be diluted by glutamic acid from other sources.

These estimates indicate that half of the glycine or of the glutamic acid of liver GSH of rats and rabbits is replaced in less than 4 hours.

*dl-Glutamic Acid*—It is reasonable to assume that GSH and protein behave in the same manner with regard to their interaction with *dl*-glutamic acid, although this is known (6) to be incompletely metabolized.

The isotope concentration in the non-protein nitrogen fraction of the intestine was about 4 times higher than that in the liver (Table II), differing from the results after feeding isotopic glycine. Furthermore, the isotope concentration of the GSH was in liver equal to, and in the intestine lower than that of the corresponding non-protein nitrogen fraction. This finding might be connected with the feeding of the racemic amino acid.

The protein glutamic acid contained less than half the isotope concentration of the GSH total nitrogen. We have not been able to determine the isotope concentration of the GSH glutamic acid in rat livers, but there can be little doubt that, since glutamic acid itself was fed, the concentration of  $N^{15}$  in GSH glutamic acid is at least twice as high as that of the total GSH nitrogen. Even in the ammonia experiments around 70 per cent of the total isotope of GSH was present in glutamic acid.

#### SUMMARY

Isotopic ammonia was administered to rabbits and *dl*-glutamic acid labeled with  $N^{15}$  to rats. The animals were killed after 2½ and 2 hours respectively. Appreciable amounts of isotope were found in both experiments in the GSH of the liver and intestine. This finding supports the conclusion drawn on the basis of experiments with isotopic glycine that GSH is rapidly metabolized and interacts more rapidly with dietary nitrogen than the protein of the same tissue. The half lifetime of the GSH in the livers of the rats and rabbits is calculated to be about 2 to 4 hours.



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# THE IN VITRO FORMATION OF PHOSPHOLIPID BY BRAIN AND NERVE WITH RADIOACTIVE PHOSPHORUS<sup>\*</sup> AS INDICATOR

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*In vivo* experiments with radioactive phosphorus have clearly established that liver and small intestine are the sites of the most intense phospholipid activity (1-4). Phospholipid activity in the brain, on the other hand, is very low (4-7). These differences raised the question whether nerve tissue can synthesize phospholipid independently or whether it acquires phospholipids from the plasma only after they have been formed by the more active tissues. The demonstration of the formation of phospholipid by liver slices *in vitro* (8) provided the necessary tool for answering this question. This procedure has been employed here to investigate the formation of phospholipid by brain and nerve that had been removed from the influence of other tissues.

## EXPERIMENTAL

Rats of the following weights were chosen for study: 15 gm. (7 days old), 50 gm., and 200 gm. The weights of the animals in each group did not vary by more than 5 per cent. Both sexes were used. The forebrain, as previously defined (6), was the only part of the brain used in the experiments described below. Slices were prepared by cutting this brain division transversely; *i.e.*, at right angles to the longitudinal fissure. The slices varied from 0.2 to 0.5 mm. in thickness. Since the slices were fragile, they were not suspended in Ringer's solution before being weighed; they were placed directly on a small square of waterproof cellophane and then weighed. Approximately 300 mg. of slices<sup>1</sup> were then transferred to a 50 cc. Erlenmeyer flask containing 5 cc. of the radioactive Ringer's solution.<sup>2</sup>

Homogenized preparations of brain were prepared after the manner of

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<sup>1</sup> In the case of the 15 gm. rat it was necessary to use the slices prepared from at least two forebrains in order to obtain 300 mg. of suitable slices; in the 50 and 200 gm. rats one forebrain was usually sufficient to give the necessary amount of slices.

<sup>2</sup> In order to make direct comparisons from experiment to experiment the ratio of slices to radio-Ringer's solution was kept as constant as possible. That is, from 280 to 320 mg. of brain slices or homogenate were used in each run. In most of the runs 290 to 310 mg. of brain tissue were used.

Potter and Elvehjem (9). The brains were ground in Ringer's solution in a stainless steel apparatus similar to that described by Colowick, Welch, and Cori (10). The amount of Ringer's solution used was sufficient to give a concentration of 300 mg. of brain per cc. of homogenate. 1 cc. of this homogenate was added to 4 cc. of the radio-Ringer's solution.

The bicarbonate-Ringer's solution was prepared according to the method of Krebs and Henseleit (11). The total amount of inorganic phosphorus used in each bath, whether it contained slices or homogenate, was always 0.18 mg. of phosphorus in the form of  $\text{KH}_2\text{PO}_4$ . From 1.25 to 2.50 microcuries<sup>3</sup> of radioactive phosphorus were present in each sample.

*Extraction of Phospholipids from Brain*—The method of extraction employed in these experiments was essentially the same as that described by Fishler *et al.* (8). However, since it was found more difficult to remove contaminating inorganic phosphorus from the phospholipids extracted from brain than phospholipids extracted from liver (considerably greater amounts of  $\text{P}^{32}$  were used in these brain experiments than in liver experiments), certain modifications were introduced in the method of extraction. These modifications consisted in repeated dilution of the radioactive inorganic phosphorus with non-radioactive inorganic phosphorus at every possible step. Thus, (1) 1 cc. of a saturated solution of non-radioactive  $\text{Na}_2\text{HPO}_4$  was added to the flasks after removal from the thermostat; (2) 1 cc. of saturated  $\text{Na}_2\text{HPO}_4$  solution was added to the combined alcohol-ether extracts just before concentration; (3) the ether solutions containing the phospholipid were shaken with 10 drops of saturated  $\text{Na}_2\text{HPO}_4$  solution containing excess solid  $\text{Na}_2\text{HPO}_4$ . The latter step was found very effective in removing traces of radioactive inorganic phosphorus.

The completeness of separation of radioactive inorganic phosphate from phospholipid was determined by a 0 time experiment, in which the phospholipids were extracted from the tissue immediately after the slices, or homogenate, were added to the radioactive Ringer's solution.

*Determination of Inorganic Phosphorus of Forebrain*—The whole forebrains of 15, 50, and 200 gm. rats were ground with ice-cold trichloroacetic acid with acid-washed sand in a glass mortar. The mixture was filtered, and the color, which was developed according to King's method (12), measured with a photoelectric colorimeter.

## Results

*Formation of Radioactive Phospholipid by Brain Slices*—The formation of radioactive phospholipid by surviving brain slices obtained from 15, 50, and 200 gm. rats is shown in Table I. The results are expressed as the percentage of the added inorganic radiophosphorus recovered as radio-

<sup>3</sup> 1 microcurie =  $4 \times 10^5$  radioactive counts as measured on the Geiger counter.

phospholipid per gm. of wet and dry tissue. These values measure only the radiophospholipid formed from inorganic radiophosphorus of the Ringer medium. Since inorganic phosphorus and various phospholipid intermediates present in the tissue itself are undoubtedly converted to phospholipid, these values represent but a fraction of the total amount of phospholipid formed by the slices. The data show that a greater percentage of the radiophosphorus of Ringer's solution is converted to radio-

TABLE I

*Formation of Radioactive Phospholipid by Brain*

All values are expressed as per cent of the labeled phosphorus of the bath incorporated into phospholipid per gm. of tissue. All the values recorded for 1, 2, and 4 hours have had the average 0 time value subtracted.

Time interval	Brain slices						Brain homogenate					
	15 gm. rat		50 gm. rat		200 gm. rat		15 gm. rat		50 gm. rat		200 gm. rat	
	Wet	Dry*	Wet	Dry*	Wet	Dry*	Wet	Dry	Wet	Dry	Wet	Dry
hrs												
0	0.0067	0.054	0.0058	0.028	0.0093	0.043	0.0047	0.038	0.023	0.11	0.027	0.13
0	0.0047	0.038	0.011	0.067	0.013	0.061	0	0	0	0	0.015	0.070
0	0.0067	0.054	0.0035	0.017	0.0096	0.045	0.0093	0.075	0	0	0.035	0.16
Average	0.0060	0.049	0.0078	0.037	0.011	0.050	0.0050	0.038	0.007	0.04	0.026	0.12
1	0.32	2.6	0.29	1.4	0.15	0.70	0.13	1.0	0.064	0.27	0.075	0.24
1	0.33	2.7	0.32	1.6			0.11	0.91	0.049	0.20	0.074	0.24
1	0.34	2.7	0.25	1.2	0.21	0.96	0.10	0.89	0.056	0.23	0.086	0.29
2	0.53	4.3	0.38	1.8	0.42	2.0	0.15	1.2	0.079	0.38	0.075	0.24
2	0.64	5.1	0.51	2.4	0.51	2.4	0.16	1.3	0.074	0.36	0.088	0.30
2	0.65	5.3	0.47	2.1			0.16	1.3	0.062	0.26	0.078	0.26
4	0.70	5.7	0.52	2.5	0.47	2.2	0.22	1.8	0.097	0.46	0.12	0.57
4	0.84	6.8	0.46	2.2	0.57	2.7	0.21	1.7	0.095	0.45	0.095	0.45
4	0.85	6.9	0.55	2.6	0.52	2.4	0.20	1.6	0.082	0.36	0.083	0.39

\* The water content of the forebrain of 15, 50, and 200 gm. rats is 87.6, 79.2, and 78.6 per cent, respectively (13)

phospholipid in the brain slices obtained from the 15 gm. rat than in those from the 50 and 200 gm. rats. There is little difference in the values obtained from the 50 and 200 gm. rats.

*Formation of Radioactive Phospholipid by Brain Homogenate*—Table I records the formation of radioactive phospholipid by brain homogenates. The results are expressed in the same manner as for the brain slices. It is seen that the amount of radiophosphorus of Ringer's solution that is converted to radiophospholipid is greater in the homogenate from the 15 gm. rat than in that from 50 and 200 gm. rats. As in the case of brain

slices, there is little difference between the 50 and 200 gm. rats. It should be noted, however, that there is considerably less conversion of Ringer's solution radiophosphate to radiophospholipid by brain homogenate than by brain slices. The conversion is 3 to 5 times greater in the slice than in the homogenate.

In order to determine whether metal from the homogenizer affected the recovery of radiophospholipid, homogenized samples of forebrain were prepared by mashing brains in an agate mortar. These samples were then treated in the same manner as those described above. The recoveries obtained with the use of the agate mortar were in close agreement with those obtained with the stainless steel homogenizer.

*Comparison of Formation of Radioactive Phospholipid by Brain "Mince" and Brain Homogenate*—In this experiment a mince was prepared by

TABLE II

*Formation of Radioactive Phospholipid by Brain Mince and Brain Homogenate Prepared from 15 Gm. Rats*

All values are expressed as per cent of the labeled phosphorus of the bath incorporated into phospholipid per gm. of wet tissue.

Time interval	Homogenate	Mince
hrs.		
1	0.15	0.14
1	0.16	0.10
1	0.16	0.11
4	0.23	0.33
4	0.23	0.24
4	0.21	0.26

foreing brains of 15 gm. rats through a brass screen. Shreds of brain tissue 5 to 10 mm. long and 0.15 to 0.20 mm. square were thus prepared. Approximately 300 mg. of these shreds were transferred to a flask containing 5 cc. of radio-Ringer's solution. The brain homogenate was prepared from 15 gm. rats as described above.

The formation of radiophospholipid by these two brain preparations was compared (Table II). Formation of radiophospholipid by brain shreds is approximately equal to that by homogenate despite the fact that the shred represents a tissue preparation with considerably greater organization than the homogenate. It would appear that the contact between the brass screen and brain shreds was too brief to cause poisoning of this preparation.

*Formation of Radioactive Phospholipid by Dog Nerve*—In order to determine whether peripheral nerve *per se* is capable of synthesizing phos-

pholipid from inorganic phosphorus, the following experiment was conducted. A dog was killed by an intercardiac injection of amytal (30 mg. per kilo) and both sciatic nerves removed as quickly as possible and placed in a bath of non-radioactive Ringer's solution. The sciatic nerve was first stripped free of all adipose and connective tissue; then this large nerve trunk was teased with a needle into smaller nerve bundles about 1 mm. thick. These bundles were cut into pieces 2 to 3 mm. long. 300 mg. of this preparation were blotted with filter paper, weighed, and then transferred to 5 cc. of radio-Ringer's solution. For control purposes, the adipose-connective tissue surrounding the nerve was cut into small fragments and similarly treated. The conversion of radiophosphate of Ringer's solution to radiophospholipid by nerve was found to be consider-

TABLE III

*Formation of Radioactive Phospholipid by Dog Sciatic Nerve*

All values are expressed as per cent of the labeled phospholipid of the bath incorporated into phospholipid per gm. of tissue. The 0 time values are not subtracted.

Time interval <i>hrs.</i>	Nerve		Adipose-connective tissue per gm. wet tissue
	Wet tissue	Dry tissue*	
0	0.006		0.008
0	0		0
4	0.63	1.6	0.036
4	0.72	1.8	0.026
4	0.44	1.1	

\* The water content of the nerve was assumed to be 60 per cent.

able (Table III). In respect to dry weight, which is probably the best method of comparison, the formation of radiophospholipid by the sciatic nerve of the dog is of the same order of magnitude as that by the brain homogenate prepared from the 15 gm. rat. Since the formation by adipose-connective tissue was negligible, it is safe to conclude that the formation of radiophospholipid found in the case of the nerve was not due to neighboring tissue surrounding the nerve. These experiments show that the nerve process, separated from the nerve cell body, can form phospholipid from inorganic phosphorus. The synthetic capacity of the neurilemma cells or neuroglia would have to be very great to account for the radiophospholipid formed by nerve.

*Decomposition of Phospholipid in Brain Homogenate*—15 and 200 gm. rats were injected intraperitoneally with radioactive phosphorus and killed several days later; in this way radioactive phospholipid was de-

posited in the brain. A homogenate was prepared from the forebrains of these rats. 1 cc. of this homogenate, containing 300 mg. of the original brain tissue, was placed in 4 cc. of non-radioactive Ringer's solution and incubated as above. Phospholipids were extracted as above and their radioactivity determined. The values are recorded under the heading of "labeled phospholipid" in Table IV.

Total phospholipid was measured by oxidative procedures (14). Since in these experiments the amount of phospholipid available in each sample was not sufficient for both measurements (labeled and total), the values shown for labeled phospholipid and total phospholipid in Table IV represent *separate* experiments. In computing the percentage of phospholipid remaining after 1, 2, and 4 hours, the average of the three 0 time values

TABLE IV  
*Decomposition of Phospholipid in Brain Homogenate*

The average 0 time value has been called 100 and all other values have been calculated relative to this. The figures in parentheses are the averages.

Time interval  hrs.	15 gm. rat		200 gm. rat	
	Labeled phospholipid remaining	Total phospholipid remaining	Labeled phospholipid remaining	Total phospholipid remaining
0	98, 105, 96 (100)	109, 99, 94 (100)	97, 94, 108 (100)	106, 97, 97 (100)
1	106, 90, 91 (96)	99, 92, 93 (95)	90, 92, 93 (92)	88, 87 (88)
2	94, 88, 88 (90)	81, 84, 87 (84)	92, 90, 94 (92)	92, 87, 87 (89)
4	85, 93, 88 (89)	74, 83, 82 (80)	96, 92, 83 (90)	85, 91, 88 (88)

For experimental details see the text.

(Table IV) was taken as 100 and the other values expressed as a percentage of this.

The amount of phospholipid found after 4 hours was about 10 to 15 per cent less than that present at 0 time. This was observed for both total phospholipid as measured by the oxidative procedure and labeled phospholipid measured by radioactivity. Most of the decrease took place in the 1st hour. In contrast with these findings, it should be noted that practically no breakdown was observed in liver slices (8) during the 1st hour, whereas a breakdown to the extent of 30 per cent was found after 6 hours. No significant differences were observed in the breakdown of phospholipid by brains of 15 and 200 gm. rats.

#### DISCUSSION

In order to make use of the measurements of radiophospholipid for comparing absolute amounts of phospholipid formed by young and old

brains, the following factors must be taken into consideration: (1) the rate of penetration of inorganic phosphorus into the brain slices, which in turn bears on the next factor, namely (2) the specific activity of the inorganic phosphorus within the brain slice. It is by no means unlikely that the differences between young and old brains in the percentages of added  $P^{32}$  recovered as phospholipid may be explained by differences in the specific activities of the inorganic phosphorus that participates in phospholipid formation within the brain preparations, without assuming a difference in the actual amounts of phospholipid formed by young and old brain preparations.

Determinations of inorganic phosphorus of the particular brain division used in this study were made in 15, 50, and 200 gm. rats; the averages of six values were respectively 0.022, 0.037, and 0.046 per cent. If it be assumed that inorganic phosphorus outside the tissue preparation comes into instantaneous equilibrium with the inorganic phosphorus inside this system, then it can be shown that the difference in specific activities of the inorganic phosphorus in young and old brain is not sufficient to explain the more than 2-fold difference found in the percentages of  $P^{32}$  recovered as phospholipid in the 15 and 200 gm. rats. Hence, if a rapid equilibrium does occur under the conditions of this experiment, it may be assumed that the younger brain forms a greater amount of phospholipid.

It is known, however, that inorganic phosphorus penetrates the central nervous system *in vivo* very slowly (4, 7, 15-17). If we assume this to be the case *in vitro* (i.e. under the conditions of the present experiment) and that equal but small amounts of radioactive inorganic phosphorus enter the brain slices of young and old rats, then the ratio of specific activities of the inorganic phosphorus in the young (15 gm.) and old (200 gm.) brains would be approximately 2, a value that is in close agreement with the 2- to 2.5-fold difference observed in the percentages of  $P^{32}$  recovered as phospholipid in the brain slices of 15 gm. and of 200 gm. rats. Hence, if a *slow* as well as equal penetration does occur, it may be assumed that the younger brain does not form more *total phospholipid* than the older brain.

The samples of radiophosphorus used in this investigation were prepared in the Berkeley cyclotron by members of the Radiation Laboratory under the direction of Professor E. O. Lawrence, to whom our thanks are due. The assistance of Dr. C. Entenman in the determination of phospholipids by the oxidative procedure is gratefully acknowledged.

#### SUMMARY

1. The *in vitro* formation of phospholipid by excised brain of young and old rats and by excised nerve of the dog is demonstrated. Formation of phospholipid was observed in brain homogenate as well as in brain slices.



2. The formation of phospholipid by brain of young and old rats is compared.

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## PYRUVIC ACID

### I. COLLECTION OF BLOOD FOR THE DETERMINATION OF PYRUVIC AND LACTIC ACIDS\*

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The accurate determination of pyruvic and lactic acids is a matter of considerable theoretical and practical importance. These acids play a rôle in the metabolism of carbohydrates, and they are found in increased concentration in the blood in conditions of thiamine deficiency. Recently we suggested (1) the determination of the ratio of lactic to pyruvic acid in the blood as a measure of oxidative conditions in tissues, and we have applied it to various studies now in progress dealing with muscular exercise and the reaction of human subjects to high altitude.

Large and indeterminate losses, which make the results valueless, may occur during collection and subsequent handling of blood samples. Until recently, samples of blood to be used for this determination have been collected with syringes and then transferred to bottles containing oxalate. Wilkins, Weiss, and Taylor (2) were the first to show that pyruvic acid disappears from such samples. This was confirmed by Bueding and Wortis (3), who suggested the use of sodium monoiodoacetate for the "stabilization" of the pyruvate. Bueding and Goodhart (4) have recently recommended the addition of 1 per cent each of iodoacetate and fluoride to samples of oxalated blood.

Consistent results, without apparent loss or gain of pyruvic acid during collection of the sample, can be obtained by using a very simple procedure. Blood is withdrawn from the vein by means of 2 or 5 cc. syringes,<sup>1</sup> and the volume is adjusted to the mark, after which the sample is expelled in a fine stream through the needle into a measured volume of precipitant. The procedure eliminates not only the use of a sample bottle containing oxalate

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<sup>1</sup> It is not generally realized that the ordinary glass syringes are instruments of considerable precision. Their use is not mentioned in any of the standard text-books or manuals on analytical chemistry. In the past, syringes have been used either as a part of apparatus for the rapid automatic measurement of liquids, or their construction has been modified in order to increase the accuracy. They should find wider application in biochemical analysis.

and fluoride, but also a pipette. The entire operation is accomplished in 30 to 45 seconds. This method has been in use many years in our laboratory for other types of determinations (5), and in these and the determination of pyruvic acid the maximum error has never been greater than  $\pm 1.5$  per cent. This is within the limits of error of the present methods for pyruvic and lactic acids.

As far as we are aware, the pyruvic and lactic acid content of freshly drawn untreated blood has never been determined. In this paper we shall discuss the effect of various factors, such as stasis during collection of the sample and the presence of iodoacetate, oxalate, fluoride, and other salts, on the pyruvic acid content of such blood.

#### EXPERIMENTAL

The subject rested in an easy chair 1 hour before the beginning of the experiment. The majority of experiments was not begun until at least 3 hours after a meal.

The sample was withdrawn with a minimum of stasis before collection; the *tourniquet* (a soft rubber tube,  $\frac{3}{8}$  inch in diameter) was removed immediately after entry of the needle into the vein. This precaution was observed throughout, despite the repeated finding that moderate stasis during the collection of the sample does not apparently affect the pyruvic and lactic acid levels. However, the subject was cautioned against clenching and opening the hand, since muscular movements may affect the results.

Blood was collected by means of carefully cleaned, dry, sterile (autoclaved) 5 cc. syringes fitted with 21 gage hypodermic needles. *Warm syringes were never used.*

The syringe was held vertically, it was tapped a few times to loosen any bubbles of air adhering to the walls, and the plunger was moved upward slowly until the blue line coincided with the 5 cc. mark. The sample was then rapidly ejected in a fine stream through the needle into 5 volumes of a cold 10 per cent solution of trichloroacetic acid contained in a cork-stoppered 50 cc. centrifuge tube. The contents of the tube were mixed immediately and then centrifuged. The tube was placed in the refrigerator and it was kept there until the time of analysis.<sup>2</sup> Pyruvic acid was determined in the clear supernatant solution by a modification of the Lu (6) method.

<sup>2</sup> It is important to keep the extract or contents of the tube cold. After several hours in a warm room, an increase of 0.05 to 0.20 mg. per cent of "pyruvic acid" (hydrazine-binding substances) is often noted.

In experiments involving the use of solutions of iodoacetate<sup>3</sup> or of other salts, about 1 cc. of the solution was drawn into the syringe. The walls were wetted by the solution, after which the air and excess of solution were expelled. In experiments involving the use of fluoride, the sample was collected in a syringe containing a weighed quantity of the salt. The approximate final concentrations of added substances were iodoacetate 0.8, potassium oxalate 0.2, sodium fluoride 0.4 per cent. The final concentrations of sodium chloride, sodium sulfate, and sodium bicarbonate were osmotically equivalent to 0.8 per cent of sodium iodoacetate.

The time required for sampling varied somewhat with each individual, due to such factors as the size of the vein, the venous pressure, position of the needle in the vein, etc. In a series of twenty determinations, from 12 to 34 seconds were required for the withdrawal of blood past the 5 cc. mark (about 6 cc.). The average time of half filling the syringe was 9.2 seconds. From 19 to 38 seconds, or an average of about 27 seconds, elapsed from the time of half filling to the time at which the sample was expelled into the precipitant.

The accuracy of measurements by means of syringes can be seen from the following data. Ten syringes, taken at random, delivered duplicate volumes of standard acid as follows: 5.05, 5.05; 5.00, 5.00; 5.05, 5.00; 4.99, 5.00; 4.93, 4.94; 5.00, 5.00; 4.98, 4.97; 4.95, 5.00; 5.00, 5.00; 4.97, 4.98 cc. The agreement of the duplicates should be noted. The extreme deviations from the average were  $\pm 1.2$  per cent. Since each syringe was numbered by the manufacturer, the results could be corrected if desired.

Blood, although quite opaque, can be measured with equal accuracy, as indicated by the following experiment. 125 mg. of dextrose were added to 100 cc. of whole blood. 5 cc. samples were precipitated by means of zinc hydroxide (7) and the sugar was determined by the Shaffer-Hartmann-Somogyi (8) method. When measured by means of a standard pipette, the sample contained 197 mg. of sugar per 100 cc. Single samples measured by ten syringes taken at random from stock contained 194, 194, 195, 194, 195, 197, 193, 195, 194, and 194 mg. per 100 cc.

If pyruvic acid only was to be determined, blood was withdrawn by means of 2 cc. "precision" (or tuberculin) syringes. The extreme deviation from the manufacturer's calibration of twelve syringes taken at random from stock, as in the case of 5 cc. syringes, was  $\pm 1.2$  per cent.

<sup>3</sup> Sodium iodoacetate solutions were prepared from the recrystallized acid either by neutralizing with sodium hydroxide, as recommended by Bueding and Wortis, or by adding slightly less than the required amount of sodium bicarbonate. In either case, the results were the same. Recrystallization of the acid appeared to be necessary, since the addition of solutions prepared from the uncrystallized acid (Eastman, No. 1371) resulted in a rapid increase of pyruvic acid.

Dr. Ancel Keys of the University of Minnesota, Minneapolis, has found (private communication) a somewhat smaller maximum deviation of  $\pm 1$  per cent.

The volumes of solution wetting the walls of 5 cc. syringes and remaining in the needle were 0.095, 0.11, 0.095, 0.11, 0.10, 0.10, 0.11, 0.097, 0.105, 0.090 cc. Again, the close agreement of the data should be noted. Because of this, in experiments involving the use of solutions it was not considered necessary to transfer a measured volume into the syringe as recommended by Bueding and Wortis. The volume of solution remaining in the syringe was assumed to be 0.1 cc. Since about 6 cc. of blood were withdrawn, the results were multiplied by  $6.0/5.9$ , or 1.02.

For the determination of lactic acid, 15 cc. of the clear supernatant solution of the precipitated sample were treated with the Van Slyke  $\text{CuSO}_4\text{-Ca(OH)}_2$  reagents, the volume was adjusted to 250 cc., and the mixture was cleared by centrifugation. Lactic acid was determined in 100 cc. aliquots<sup>4</sup> by a modification of the Shaffer method (9). The titrations were carried out with 0.0025 N iodine solution.

### Results

Before discussing the results, it should be pointed out that the method used in this investigation differs considerably from that of other workers in this field. In preceding studies, blood has been collected in a syringe and then either transferred to a bottle containing oxalate or heparin, or it has been defibrinated (2-4, 10). In every instance the composition of the blood has been altered; in no instance has the fresh unchanged blood been studied. The substance whose effect is to be determined is added to measured volumes of the prepared blood. The initial determinations, which serve as controls, are then made. All of these operations require many minutes. These procedures undoubtedly effect some change in the activities of the leucocytes and red blood cells. It is not altogether unlikely that the high concentration of salt *per se* may account for some of the effect, since it upsets the normal ionic equilibrium between cells and plasma. Furthermore, since no effort is made to prevent loss of  $\text{CO}_2$ , the blood may become considerably more alkaline. It is obvious that the changes which occur within a few minutes cannot be evaluated accurately by such a procedure.

In our experiments, frequent samples were withdrawn, usually simultaneously, from the right and left arms. The order in which the samples

<sup>4</sup> The clear solution contains much calcium trichloroacetate, which decomposes on heating, yielding base. It is therefore necessary to modify the lactic acid reagent. 1 liter of reagent should contain 50 cc. of syrupy (85 per cent)  $\text{H}_3\text{PO}_4$  and 200 gm. of  $\text{MnSO}_4\cdot 4\text{H}_2\text{O}$ .

were collected is shown in Tables I to IV. It will be noted that a "control" sample was taken before and after each set of samples in which the effect of a variable was to be determined. For example, the effect of incubation at room temperature (Table I) was determined by comparing the results from samples which were precipitated as soon as possible (less than 30 seconds after filling the syringe one-half) with those from samples

TABLE I  
*Effect of Sodium Iodoacetate on Blood*

Subject and time of sample collection	Incubation at room temperature, $\pm 25^{\circ}$	Left arm, no preservative, authors' procedure			Right arm, iodoacetate		
		Pyruvic acid	Lactic acid	Lactic acid Pyruvic acid	Pyruvic acid	Lactic acid	Lactic acid Pyruvic acid
	min	mg per cent	mg per cent		mg per cent	mg per cent	
L. P.,* 11.20- 11.35 a.m.	None	1.01	13.0	12 9	1 07	10.4	9.7
	1	1.01	Lost		1.12	14.9	13 3
	None	1.03	13.6	13.2	1.14	19.5	17.1
	2	1.01	13 4	13.3	1.18	21.0	17.8
	None	1.02	12.6	12 4	1 10	19.9	18.1
	3	1 06	13 3	12.6	1.40	26.1	18 6
E. G., 4.45- 5 07 p.m.	None	0.86	8.4	9 8	0 86	10 2	11 9
	1	0.77	7.1	9.2	0 85	7 3	8 6
	None				0 82	8 4	10 2
	2	0 76	8 7	11.5			
	None	0.73	8 7	11 9	0 88	13.8	15 7
	3	0.74	8 9	12 0			
W. H.†	None	0 89	11 7	13 2	0 95	8.8	9.3
	1	0 84	10 4	12 4	0 94	15 0	15.9
	None‡	0 94	11 3	12 0	0 91	18 9	20 1
	2	1 38	18 8	13 6	1 55	24 8	16 0
	None	1 32	17 2	13 0	1 45	18 2	12.6
	3	1 36	17 2	12 6	1 63	24 6	15 1

\* Subject ate breakfast at 9.15 a.m.

† Subject fainted after the third series of samples had been taken. Collection of the second set of samples was begun 12 minutes after the subject had regained consciousness.

‡ Subject pale, felt faint.

held 1, 2, and 3 minutes in the syringe before the contents were expelled into the precipitant. The alternately collected, immediately precipitated samples constituted the controls. The advantage of this method is that fresh blood, without any added salt, is used in every instance.

*Effect of Sodium Iodoacetate*—Samples collected in syringes containing iodoacetate, as a rule, contained slightly more pyruvic acid than those collected without preservative (Table I). Although the pyruvic acid content did not change during the 1st minute of incubation at room tem-

perature, it was definitely increased after 3 minutes of incubation. Some samples showed a slight increase of pyruvic acid after only 2 minutes of incubation. The determination of lactic acid was difficult in such samples, partly because of the high blank, and partly because of the greater variability in the results of titrations of duplicate samples.

TABLE II  
*Effect of Various Salts on Blood*

Subject	Substance added	Incubation in syringe at room temperature	Left arm			Right arm		
			Pyruvic acid	Lactic acid	Lactic acid Pyruvic acid	Pyruvic acid	Lactic acid	Lactic acid Pyruvic acid
		min	mg per cent	mg per cent		mg per cent	mg per cent	
L. P.*	None	3	0.86	7.2	8.1	0.75	8.2	10.9
	Fluoride	3	0.61	8.9	14.6	0.58	8.3	14.3
	Ovalate	3	0.72	9.6	13.3	0.71	9.4	13.2
	Iodoacetate	3	0.91	12.3	13.5	0.85	10.1	11.9
	None	3	0.78	8.6	11.0			
M. K. R.	"	None	0.80	8.3	10.4	0.76	7.8	11.3
	"	"	1.05	10.5	10.0	1.06	9.6	9.1
	Fluoride	"	0.89	8.3	9.3	0.94	10.0	10.6
	"	3	0.54	8.7	16.1	0.81	10.1	12.5
	None	None	0.98	9.8	10.0	0.90	8.6	9.6
	Ovalate	"	0.94	8.8	9.4			
	"	3	0.57	10.1	17.7	0.76	10.4	13.7
G.†	None	None	0.87	10.0	11.5			
	"	"	1.05	11.6	11.0	0.91	9.9	10.9
	Fluoride	"				0.81	9.6	11.9
	"	3				0.69	12.5	15.1
	None	None				1.03†	11.5†	11.2†
	"	"				0.98	13.4	13.7
	Ovalate	"				0.92	14.4	15.6
	"	3				0.61	11.7	19.5
	None	None				0.88	11.5	13.1

\* Subject ate breakfast at 7.30 a.m. Samples were collected from 11.35 a.m. to 12 noon

† Subject vomited after collection of fourth set of samples. Collection of last four samples was begun 23 minutes later.

‡ Subject pale, felt faint

In samples without added substances, no significant difference of the pyruvic acid content was noted between those immediately precipitated and those incubated for varying periods of time up to 3 minutes. The lactic acid content also was not apparently increased. This was further indicated by the constancy (within the limits of experimental error) of the ratio of lactic to pyruvic acid. See also the results from Subject L. P. in Table II.

*Effect of Oxalate, Fluoride, and Other Salts*—Bueding and Goodhart have shown that the addition of 1 per cent of fluoride to oxalated blood does not prevent the disappearance of pyruvic acid, although it does prevent an increase of lactic acid. This quantity of fluoride is considerably greater than that used by previous investigators. The series of determinations shown in Table II indicate a rapid loss of pyruvic acid in blood containing about 0.4 per cent of sodium fluoride. The loss appeared to be small in samples which were immediately precipitated. In accord with common experience, the lactic acid content was not measurably increased.

TABLE III

*Effect of Salts on Blood after 3 Minutes of Incubation at Room Temperature*

The values are expressed in mg. per cent.

Salt	Subject V. H.	Subject M. H., Experiment 1	Subject M. H., Experiment 2	Subject L. P.		Subject E. S.	
				Left arm	Right arm	Left arm	Right arm
None.....			0.69	0.78		0.72	
NaCl, 11.2%*.....			0.77	0.92		0.65	
None.....		0.66	0.70	0.78		0.73	
NaHCO <sub>3</sub> , 16%*.....		0.47		0.65		0.54	
None.....		0.65		0.82	0.81	0.70	0.65
Na <sub>2</sub> SO <sub>4</sub> , 18%*.....			0.60		0.82		0.59
None.....	0.75		0.63		0.95		0.69
NaCl, 25%.....	0.65				0.99		0.67
None.....	0.72				1.00		0.67

\* The use of these solutions in 5 cc. syringes yielded a final concentration in the blood osmotically equivalent to 0.8 per cent of sodium monoiodoacetate. The bicarbonate did not dissolve completely. The suspension was saturated with CO<sub>2</sub> before use. It was agitated during filling and emptying of the syringe.

The addition of 0.2 per cent of oxalate also brought about a loss of pyruvic acid, but the rate of removal was smaller than with fluoride. No apparent loss was noted in samples which were immediately precipitated. However, a considerable diminution in pyruvic acid was noted in all samples after 3 minutes of incubation. The lactic acid content was not apparently altered by keeping the samples 3 minutes at room temperature.

The effect of salts, to which reference was made above, is shown in Table III. Sodium chloride, when present in a concentration osmotically equivalent to 0.8 per cent of monoiodoacetate, definitely increased the pyruvic acid content in two experiments. In the third experiment, the concentration was slightly lower (perhaps within the limits of experimental error), 0.65, as compared with 0.72 and 0.73 in the control samples. At



the same osmotic concentration of sodium sulfate, a trend toward a lowered pyruvic acid content was noted in every experiment. The greatest losses of all were noted in the samples which contained sodium bicarbonate. The solution of bicarbonate was saturated with  $\text{CO}_2$  just before the experiments. This was done in order to minimize the possible effect of a rise of pH of the blood and to provide a means of testing the comparative effect of the bicarbonate ion. At a somewhat higher concentration of sodium chloride, with a 25 per cent solution, the pyruvic acid content appeared to be unaltered in two experiments and slightly decreased in another experiment.

TABLE IV  
*Effect of Stasis on Blood*

Subject	Stasis	Left arm			Right arm		
		Pyruvic acid	Lactic acid	Lactic acid Pyruvic acid	Pyruvic acid	Lactic acid	Lactic acid Pyruvic acid
		mg per cent	mg per cent		mg per cent	mg per cent	
L. P.	None	0.95	10.8	11.4	0.93	11.7	12.6
	Stasis	0.98	14.9	15.2	0.92	13.1	14.2
	None	1.07	14.3	13.4	1.06	13.8	13.0
	Stasis	0.97	13.7	14.1	1.06	13.8	13.0
	None	0.95	13.2	13.9	0.98	12.8	13.1
W. P. M., Experiment 1	"	1.23			1.18		
	Stasis	1.20			1.36		
	None	1.20			1.09		
	Stasis	1.03			1.11		
W. P. M., Experiment 2	None	0.87			0.89	9.8	11.0
	Stasis	0.82	10.0	12.2	0.87	10.7	12.3
	None	0.78	9.9	12.7	0.87	11.3	13.0

*Effect of Stasis*—It has long been held that asphyxial conditions favor the production of lactic acid by tissues. Such conditions greatly alter the ratio of lactic to pyruvic acid (1). It would seem, therefore, that pyruvic acid should be determined only in blood which has been collected without any stasis whatsoever. In practice, however, this is not feasible. In the majority of instances, the insertion of the hypodermic needle into the vein is accomplished in a minimum of time, and with a minimum of injury to the vein and surrounding tissue, only when the vessel has been engorged with blood by the momentary application of the tourniquet. It is particularly necessary in experiments requiring the collection of many samples at accurately spaced intervals.

In order to test the effect of stasis, unusually severe conditions were

employed. The tourniquet, instead of being applied temporarily, until the needle had been inserted into the vein, as is the custom in this laboratory, was applied for a period of 2 minutes before the collection of the sample was begun. The tourniquet remained on the arm until the collection was complete. Alternate samples of blood, which served as controls, were collected without any stasis whatsoever. The results in Table IV show that these unusual conditions affected the pyruvic and lactic acid content of the blood very slightly, if at all. Of special interest is the finding that the ratio of lactic to pyruvic acid was not significantly changed. Apparently the flow of blood was not greatly impaired by the application of the tourniquet.

### DISCUSSION

The experiments of previous workers have shown that pyruvic acid disappears rapidly from defibrinated, heparinized, or oxalated blood. In all of these procedures the blood becomes more alkaline. That the changes may be due to the presence of the oxalate or to the increased alkalinity has not apparently been recognized. Since oxalate has not been shown to have an effect on any of the intermediary reactions of carbohydrate occurring in blood, it has been assumed that the oxalate is of no consequence and that the analytical results represent the changes which occur in freshly drawn blood. The study of "stabilizing" agents so far has been limited to iodoacetate. That a similar effect, although perhaps not so pronounced, may be obtained with other salts, which do not have the specific effect of iodoacetate, has not been considered.

This study indicates that some salts bring about the disappearance of pyruvic acid while others effect an increase. Thus pyruvic acid disappears rapidly on the addition of sodium oxalate, fluoride, or bicarbonate. A similar trend is noted in samples containing sodium sulfate. On the other hand, the pyruvic acid content increases rapidly in the presence of sodium iodoacetate, and apparently more slowly at the equivalent concentration of sodium chloride. It is probable, therefore, that the temporary stabilizing effect of iodoacetate added to oxalated blood, as observed by Bueding and Wortis, is due to a balance between the reactions which increase and those which remove pyruvic acid. In the absence of any added salts, *i.e.* without the addition of anticoagulants or "preservatives," fresh blood may be held for as long as 3 minutes in a cool syringe without any indication of loss or gain of pyruvic acid.

Despite this apparent stability of the fresh untreated blood, the results of analyses are almost always lower (from 0.05 to 0.15 mg. per cent) than those obtained from samples which contain iodoacetate or iodoacetate plus

oxalate. Such a difference, immediately after withdrawal, has never been noted with any salts except the iodoacetate. The discrepancy may be due to conversion of some of the iodoacetate into keto acid, such as occurs spontaneously in solutions of trichloroacetic acid. On the other hand, it may be the result of reactions which occur *at the time of withdrawal* of the sample from the blood vessel. It is limited to this time because no apparent further change occurs for at least 1 minute thereafter. Therefore, it is due either to an extremely rapid removal of pyruvic acid from the untreated blood or to an equally rapid conversion of *preformed* precursors into pyruvic acid, catalyzed by the iodoacetate, in the "preserved" blood.

We prefer the procedure described in this paper because of its simplicity and convenience. Although measurement of the sample by means of a syringe is not as accurate as the measurement by means of a pipette, the error is within the limit of error of the analytical methods. Since the sample is measured and the proteins immediately precipitated, the errors introduced by the addition of iodoacetate and other salts are eliminated. Lactic acid is determined more accurately, and the lactic-pyruvic ratios are more constant (see, for example, the results from W. H., Table I), in the freshly collected untreated samples of blood than in those samples which contain monoiodoacetate.

#### SUMMARY

Blood is withdrawn from the vein by means of a cool, dry, clean 2 or 5 cc. syringe. The volume of the sample is adjusted to the mark and the contents are expelled in a fine stream through the needle into 5 volumes of trichloroacetic acid. The entire operation requires from 30 to 45 seconds. The error of measurement of blood by means of syringes is within the limits of error of the methods for the determination of pyruvic and lactic acids.

Application of a tourniquet for a period of 2 minutes, and continued application during collection of the sample from the vein, did not noticeably affect the results. Although moderate stasis has no apparent effect, it is recommended that the tourniquet be released as soon as the needle has entered the vein.

Fresh untreated blood, when held at room temperature in cool syringes for varying periods of time up to 3 minutes, did not gain or lose pyruvic acid.

Sodium bicarbonate, sodium fluoride, sodium oxalate, or sodium sulfate when added to blood brought about a loss of pyruvic acid. The rate of loss was greatest with the bicarbonate and least with the sulfate. On the other hand, a rapid increase of keto acid was noted in the samples to which sodium monoiodoacetate was added. In two out of three experiments, a distinct increase of pyruvic acid was noted following the addition of

sodium chloride. The apparent "stabilizing" effect of sodium fluoride and iodoacetate added to oxalated blood is probably due to a balance between many reactions, some of which result in a loss and others in a gain of pyruvic acid. The simple and convenient procedure described by the authors completely eliminates the errors resulting from the addition of salts.

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# FACTORS AFFECTING THE RIBOFLAVIN CONTENT OF THE LIVER

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Although the concentration of riboflavin in animal tissue tends to follow the intake, the available data are quite inadequate for establishing a consistent relationship which might aid in anticipating general requirements or the demand imposed by particular phases of metabolism. Physical exercise (1) and high fat diets (2) have been reported to increase the demand, and recorded data show that the riboflavin concentration in the liver may vary more than 100 per cent when calculated either on the basis of fresh tissue or the dry basis. No consistent correlation was found, however, between the concentration in muscle tissue and blood and the intake in rats (3) or humans (4). It is assumed, however, that a certain minimum concentration in the tissues is essential for maintaining life, but there has been no recorded evidence of a transient "mobilization" in the liver to meet immediate and particular requirements of assimilation and metabolism.

Since riboflavin is essential for physiological oxidation and because primary or partial oxidation of fatty acids, for example, is believed to be localized in the liver, a series of studies was designed to determine whether temporary variations in riboflavin concentration were induced under the stimulus of digestion and assimilation. The particular objective was to determine the riboflavin concentration in the liver following the ingestion of food and injection of riboflavin, thiamine, pyridoxine, and pantothenic acid following previous impoverishment in the animal of each of these factors.

## EXPERIMENTAL

The experimental plan involved the depletion of large groups of white rats, 23 to 25 days old at weaning, of the particular factor whose influence it was desired to study; substantially 400 animals were used for obtaining the records presented here. The basal ration was identical for all groups, depletion of a particular factor being accomplished by omission of that substance from the primary supplements furnished as a known dosage per rat per day (Table I). The basal ration consisted of vitamin-free casein (Labco) 20, sucrose 69, hydrogenated vegetable oil (Crisco)

3, Salt Mixture 40 (5) 4, powdered agar-agar 2, and medicinal cod liver oil 2 parts. Various groups of animals were prepared by depleting them of riboflavin, thiamine, vitamin B<sub>6</sub>, or pantothenic acid; other groups received none of the primary supplements for a period of 3 or 4 weeks following weaning, it being impossible to maintain the animals for longer periods in the absence of all these factors. Still other groups were prepared for determining the influence of tissue saturation with thiamine. Such preparation involved depletion until the animals exhibited a medium degree of paralysis, at which time they received 100  $\gamma$  of thiamine orally per day for 5 days. Additional groups were used for determining the influence of an increased amount of pantothenic acid. Such animals received 50  $\gamma$  of

TABLE I  
*Supplements Furnished per Rat per Day during Depletion Periods*

Substance supplied	For riboflavin depletion	For thiamine depletion	For vitamin B <sub>6</sub> depletion	For pantothenic acid depletion
Riboflavin, $\gamma$ .....		10	10	10
Thiamine, $\gamma$ ..	12 5	0 75	12 5	12 5
Rice polish concentrate (Labco), autoclaved, pH 8.5, mg.*	100	100		
Rice polish factor 2 (Labco), mg.†			100	
" " " 2 " autoclaved, pH 11, mg.‡				100
Vitamin B <sub>6</sub> , $\gamma$ .....				10

\* Contains no vitamin B<sub>1</sub>, or riboflavin, but does contain 11 to 13  $\gamma$  of pantothenic acid and 7 to 8  $\gamma$  of vitamin B<sub>6</sub> per 100 mg.

† Contains no vitamin B<sub>1</sub>, riboflavin, or vitamin B<sub>6</sub>, but does contain 20  $\gamma$  of pantothenic acid per 100 mg.

‡ Contains no vitamin B<sub>1</sub>, riboflavin, vitamin B<sub>6</sub>, or over 0.3  $\gamma$  of pantothenic acid per 100 mg.

pantothenic acid for 13 weeks in addition to the other requisite primary supplements. Depletion of each of the factors was determined by the criteria prevailing at these laboratories (3, 6-8).<sup>1</sup>

Following appropriate preparation, the requisite number of animals were fasted for a 24 hour period prior to the administration of 1 gm. of the basal ration and supplements other than the particular one under investigation. The food was given in semifluid form by forced feeding with a blunt hypodermic needle. (A degree of fluidity of the basal ration suitable for handling in this manner was obtained by mixing 50 gm. of the dry ration with sufficient water to make a 60 ml. volume.) The vitamin factors were administered singly or in combination as hereinafter noted, usually in 100

<sup>1</sup> See also Supplee, G. C., Bender, R. C., and Kahlenberg, O. J., unpublished data.

$\gamma$  quantities, in 0.5 ml. of physiological salt solution by injection directly into the heart. This procedure avoided certain uncontrolled features such as loss through excretion, variable absorption, and other unknown elements of control incident to oral feeding or injection at other sites.

Animals ready to receive a particular test substance were divided into two major groups with an equal number of each sex. One group which received 1 gm. of food only served as the negative control; data from this group are representative of the effect accruing from previous depletion or impoverishment of a particular factor. The second group received 1 gm. of food and also the injected vitamin; the data are representative of the effect of the vitamin as manifested during the following 24 hour period. From eight to twelve animals from each test group which received neither the 1 gm. of food nor the vitamin injection were sacrificed at the end of the preliminary 24 hour fast period and the liver immediately removed for analysis. All animals in the subgroups which were fed, or fed and injected, were returned to screen bottom metal cages and supplied with water only. At intervals of 4 hours, at least four animals from each group were sacrificed and the liver immediately removed for analysis. Glycogen was determined in one lobe of the liver by the method of Good, Kramer, and Somogyi (9); the results were used for calculating the glycogen content of the whole liver. Riboflavin determinations (7) were made on the remaining portion following desiccation and extraction of total lipids. All riboflavin values are expressed as micrograms per gm. of water-fat-glycogen-free liver tissue. In some instances the glycogen, riboflavin, and lipid determinations were made on pooled samples. In the greater majority of cases, however, determinations were made on individual specimens and the results averaged. For convenience of interpretation and comparison, the available results are presented in graphical form in Figs. 1 to 5.

Figs. 1 and 2 clearly reveal that the concentration of riboflavin in the water-fat-glycogen-free liver tissue increases during digestion and assimilation even in animals impoverished of this factor as a result of receiving a riboflavin-free diet for several weeks. (The records in Fig. 1 are from animals deprived of all the water-soluble vitamins for 3 weeks, whereas the results in Fig. 2 are from animals deprived of riboflavin only for 8 weeks.) This mobilization is temporary and the accentuated concentration tends to recede to the lower level prevailing prior to the demands imposed by assimilation of the test meal. The mobilization of riboflavin in the liver of impoverished animals not injected with this factor is relatively slow, the peak concentration being reached only after 12 to 16 hours; following this period the riboflavin level decreased rapidly to the prefeeding level. The animals which received the 100  $\gamma$  injection showed a more rapid rise in the riboflavin level with less abrupt decline following the peak concen-



tration. The results from the animals receiving the 25  $\gamma$  injection are analogous to those from the animals injected with the higher amount, but of somewhat lower magnitude.

It will be recalled that in each instance the data presented in Figs. 3, 4, and 5 were obtained from animals receiving 10  $\gamma$  of riboflavin per day during the depletion of one or another of the factors under investigation, and accordingly extreme riboflavin impoverishment did not prevail.

The data from the animals impoverished of thiamine to the paralytic state (Fig. 3, Group 1) show only a slight riboflavin mobilization tendency in the liver during digestion; this tendency was not increased by the

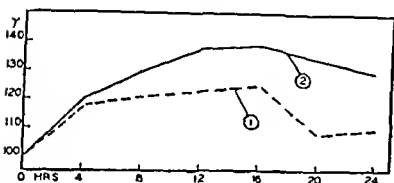


FIG. 1

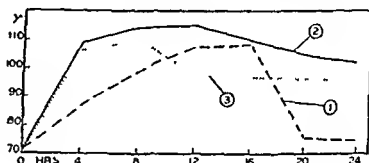


FIG. 2

FIG. 1. Riboflavin concentration in the liver as influenced by the progress of assimilation and multiple injection of vitamin factors. All animals were fed 1 gm. of food at the beginning of the 24 hour observation period; supplements during the depletion period, none; 100  $\gamma$  each of riboflavin, thiamine, vitamin B<sub>6</sub>, and pantothenic acid were injected. Riboflavin is expressed as micrograms per gm. of lipid-glycogen-water-free liver tissue. The average weight of the animals was 47.3 gm. Curve 1, controls, not injected; Curve 2, injected.

FIG. 2. Riboflavin concentration in the liver as influenced by the progress of assimilation and injection of riboflavin. All animals were fed 1 gm. of food at the beginning of the 24 hour observation period; supplements during the depletion period, 12.5  $\gamma$  of thiamine and 100 mg. of autoclaved (pH 8.5) rice polish concentrate; 100  $\gamma$  and 25  $\gamma$  of riboflavin were injected. Riboflavin is expressed as micrograms per gm. of lipid-glycogen-water-free liver tissue. The average weight of the animals was 53 gm. Curve 1, controls, not injected; Curve 2, 100  $\gamma$  of riboflavin injected; Curve 3, 25  $\gamma$  of riboflavin injected.

injection of 100  $\gamma$  of thiamine. The magnitude of increase, 8 hours after feeding, in riboflavin concentration in the livers of the animals which had previously received 100  $\gamma$  of thiamine per day orally for 5 days is markedly accentuated in comparison with the thiamine-impoverished animals; the injection of 100  $\gamma$  did not greatly increase the riboflavin level above that of the controls (Fig. 3, Group 2).

The data from the pantothenic acid studies (Fig. 4) yield riboflavin concentration curves which seem to show a direct interacting influence between these factors in the animal body. The pantothenic acid-impoverished animals (Fig. 4, Group 1) showed no evidence of an increase in

riboflavin concentration in the liver during digestion and assimilation. However, the injection of 100  $\gamma$  of pantothenic acid at the time of feeding caused a slight elevation in riboflavin level which prevailed throughout the 24 hour observation period.

The results from the animals (Fig. 4, Group 2) receiving 50  $\gamma$  of pantothenic acid for 13 weeks in addition to the usual supplements are substan-

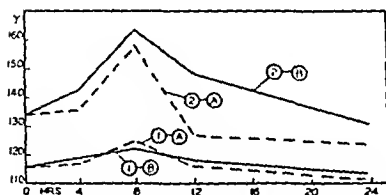


FIG. 3

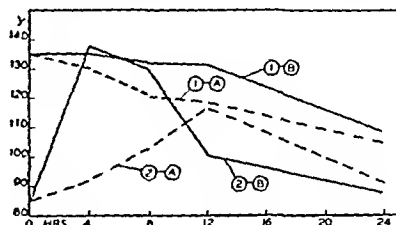


FIG. 4

FIG. 3. Riboflavin concentration in the liver as influenced by the progress of assimilation and injection of thiamine. All the animals of Group 1 were fed 1 gm. of food at the beginning of the 24 hour observation period; supplements during depletion, 10  $\gamma$  of riboflavin, 0.75  $\gamma$  of thiamine, and 100 mg. of autoclaved (pH 8.5) rice polish concentrate; 100  $\gamma$  of thiamine were injected. The average weight of the animals was 50.3 gm. The animals in Group 2 received the same treatment as those in Group 1 but with the exception that 100  $\gamma$  of thiamine were supplied orally each day for 5 days preceding the 24 hour fast prior to the observation period. The average weight of the animals was 64.2 gm. Riboflavin is expressed as micrograms per gm. of lipid-glycogen-water-free liver tissue. Curve A, controls, not injected; Curve B, 100  $\gamma$  of thiamine injected.

FIG. 4. Riboflavin concentration in the liver as influenced by the progress of assimilation and injection of pantothenic acid. All the animals in Group 1 were fed 1 gm. of food at the beginning of the 24 hour observation period; supplements during depletion, 10  $\gamma$  of riboflavin, 12.5  $\gamma$  of thiamine, 10  $\gamma$  of vitamin B<sub>6</sub>, and 100 mg. of autoclaved (pH 11) rice polish factor 2; 100  $\gamma$  of pantothenic acid were injected. The average weight of the animals was 79.3 gm. The animals in Group 2 received the same treatment as those in Group 1 but with the exception that 50  $\gamma$  of pantothenic acid were supplied daily in addition to the other supplements for 13 weeks prior to the observation period. The average weight of the animals was 152.5 gm. Riboflavin is expressed as micrograms per gm. of lipid-glycogen-water-free liver tissue. Curve A, controls, not injected; Curve B, 100  $\gamma$  of pantothenic acid injected.

tially different from those from the pantothenic acid-impovertished animals. The animals in Group 2 had an average weight more than twice that of the animals in any of the other groups. Inasmuch as the riboflavin supplement of 10  $\gamma$  per day was maintained as a constant dosage irrespective of depletion time and final weight (groups in Figs. 1 and 2 excepted), the relatively lower initial concentration in the liver is probably explained by the greater tissue demand of the larger animals. It will be noted, however, that the

concentration increases to a peak level in the controls 12 hours after feeding and declines thereafter (see the similar pattern for controls on the riboflavin-free ration, Fig. 2). Injection of 100  $\gamma$  of pantothenic acid caused a more rapid (and somewhat phenomenal) rise in riboflavin concentration notwithstanding the limited supplementation of 10  $\gamma$  per day than was manifested by any of the other groups, even including those injected with 100  $\gamma$  of riboflavin (Fig. 2). An equally abrupt decrease followed 8 hours after injection and feeding. This particular reaction pattern indicates a specific influence of pantothenic acid in mobilizing riboflavin in the liver incident to the functional demands imposed by digestion and assimilation.

The results from the vitamin B<sub>6</sub> studies (Fig. 5) did not show extreme variations in riboflavin concentration in the liver in either the control or the injected animals; slight evidence of a temporary increase was shown by both groups 12 hours after feeding.

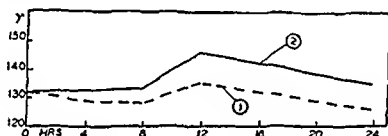


FIG. 5. Riboflavin concentration in the liver as influenced by the progress of assimilation and injection of vitamin B<sub>6</sub>. All animals were fed 1 gm. of food at the beginning of the observation period; supplements during the depletion period, 10  $\gamma$  of riboflavin, 12.5  $\gamma$  of thiamine, and 100 mg. of rice polish factor 2; 100  $\gamma$  of vitamin B<sub>6</sub> were injected. Riboflavin is expressed as micrograms per gm. of lipid-glycogen-water-free liver tissue. The average weight of the animals was 62.6 gm. Curve 1, controls, not injected; Curve 2, 100  $\gamma$  of vitamin B<sub>6</sub> injected.

The data presented illustrate the results obtained by methods designed to determine the influence of particular vitamin entities and their interaction on certain basic phenomena involved in metabolic processes. Although riboflavin is slowly lost through the excretions even on an impoverished dietary, the data lead to the conclusion that an involuntary mechanism excited by the ingestion of food induces a temporary mobilization of this factor in the liver to meet functional demands. Among the vitamin factors included in this study, pantothenic acid appears to have a more specific and direct effect upon those processes which cause this mobilization than does thiamine or pyridoxine. However, the evidence also indicates that thiamine, significantly but more indirectly, is also involved in maintaining this function.

#### SUMMARY

1. Injection of riboflavin directly into the blood stream causes an immediate increase in concentration in the liver.

2. The riboflavin concentration in the liver increases during digestion and assimilation, being mobilized therein presumably from other tissues; this transient concentration takes place even in animals whose tissue stores have been impoverished by a prolonged riboflavin-free dietary.

3. The mobilization of riboflavin in the liver during digestion and assimilation, by thiamine-depleted animals, is relatively slight. Thiamine replenishment of depleted tissues by oral feeding brings about a restoration of the riboflavin-mobilizing function.

4. Pantothenic acid appears to have a direct and specific function in the mechanism which causes the mobilization of riboflavin in the liver following ingestion of food.

5. The influence of vitamin B<sub>6</sub> on the concentration of riboflavin in the liver was found to be relatively slight in comparison with the apparent influence of the other factors studied.

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## ISOLATION OF MESOLANTHIONINE FROM VARIOUS ALKALI-TREATED PROTEINS\*

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In a previous publication (2) was described the isolation from  $\text{Na}_2\text{CO}_3$ -treated wool of a new sulfur-containing amino acid, which was named lanthionine.<sup>1</sup> The composition, chemical behavior, and properties of this compound pointed definitely to a thio ether amino acid having the structure,  $\text{HOOC}\cdot\text{CH}(\text{NH}_2)\cdot\text{CH}_2\cdot\text{S}\cdot\text{CH}_2\cdot\text{CH}(\text{NH}_2)\cdot\text{COOH}$ . That this formula represents the correct structure has been established by synthesis of the compound by du Vigneaud and Brown (3). This amino acid was isolated from an acid hydrolysate of wool previously boiled for 1 hour with a 2 per cent solution of  $\text{Na}_2\text{CO}_3$ . It separated in the form of sparingly soluble six-sided plates having a characteristic triangular appearance and was optically inactive. In addition to these crystals, there was also isolated about an equal amount of other crystals having the properties of a thio ether amino acid, which differed from the inactive lanthionine with respect to crystalline form and solubility, but had the same percentage of N. It was suggested that this compound might be an optical isomer. These crystals are discussed in the following paper (4). Recently, Brown and du Vigneaud (5) have synthesized the two optically inactive forms of lanthionine, the meso and the racemic, and have resolved the *dl*-lanthionine into the dextro and levo forms. The triangular crystals obtained from the  $\text{Na}_2\text{CO}_3$ -treated wool were found to be identical with the synthetic mesolanthionine.

The isolation of lanthionine from wool raised the question whether other proteins than wool or the fibrous keratins in general would yield thio ether amino acids. The present paper describes the isolation of lanthionine from human hair, chicken feathers, and lactalbumin by following essentially the same procedure as was used in the case of wool. Higher yields of lanthionine were obtained from hair than from wool. 100 gm of hair yielded 25 gm. of lanthionine. Considerably smaller yields were

\* A preliminary note on the isolation of lanthionine from hair, feathers, and lactalbumin has been published (1)

<sup>1</sup> The authors wish to take advantage of this opportunity to acknowledge the suggestion of Dr Vincent du Vigneaud in the naming of this thio ether amino acid. We regret that by oversight this acknowledgment was not made in our first paper

obtained from feathers (0.2 gm.). This is doubtless to be accounted for by the fact that the feathers were obtained from young birds and were not mature. The quills still retained a considerable proportion of the soft, pulpy tissue. The amount obtained from lactalbumin (0.2 gm.) was also small compared with that obtained from wool and hair. This was to be expected because of the relatively small amount of sulfur in this protein. It seems probable that lanthionine may be similarly obtained from most proteins which yield cystine on acid hydrolysis. In this connection it is of interest to note that du Vigneaud, Brown, and Bonsnes (6) have recently isolated lanthionine from alkali-treated insulin by following the same procedure as that used in its isolation from wool. That the reaction during the pretreatment of the proteins with  $\text{Na}_2\text{CO}_3$  solution represented a general alkali effect and was not due to some influence peculiar to  $\text{Na}_2\text{CO}_3$  was shown by the isolation of lanthionine from wool that had been boiled for 1 hour with 0.1 N NaOH, or with 2 per cent  $\text{Na}_2\text{S}$  solution, previous to acid hydrolysis. When higher concentrations of alkali were used, for example normal NaOH, all of the wool was dispersed, and hydrolysis of the product yielded cystine but no lanthionine.

Since the first publication, some additional data have been obtained on the relative stability of lanthionine toward alkali. Under comparable conditions, it is more stable than cystine, but less so than methionine. When lanthionine is boiled with normal NaOH containing a small amount of lead acetate, the solution begins to darken in about 3 minutes and at the end of 10 minutes there is a definite precipitation of lead sulfide. When cystine is similarly treated, the solution darkens almost at once and yields a precipitate of lead sulfide in 30 seconds. Methionine, on the other hand, shows no darkening from sulfide formation, even after being boiled for 3 minutes.

It is of interest to note that although there is no evident decomposition when lanthionine is boiled as described for 3 minutes with normal NaOH the addition of a drop of salicylaldehyde in the cold brings about a copious precipitate of lead sulfide as soon as the solution reaches the boiling point. This catalytic effect of salicylaldehyde was first shown by Clarke and Inouye (7) when working on the effect of alkali on cystine. Methionine shows no darkening after being boiled for 3 minutes under similar conditions.

#### EXPERIMENTAL

*Isolation of Lanthionine from Human Hair*—100 gm. of human hair washed with cold water were boiled for 1 hour with a 2 per cent solution of  $\text{Na}_2\text{CO}_3$ . The  $\text{Na}_2\text{CO}_3$ -treated hair was then collected on a double layer of cheese-cloth, washed with water, and suspended in 200 cc. of concen-

trated HCl. After the mixture was boiled for 15 hours, the lanthionine was isolated from the acid hydrolysate according to the procedure previously described for its isolation from wool (3). The yield of mesolanthionine was 2.5 gm. There was also obtained an equal amount of an isomeric lanthionine.

*From Chicken Feathers*—100 gm. of feathers from young chickens (friers) were treated in the same manner as described for hair. There were isolated from the hydrolysate of the  $\text{Na}_2\text{CO}_3$ -treated feathers 0.25 gm. of lanthionine and 0.3 gm. of the isomeric compound.

*From Lactalbumin*—The lactalbumin was prepared from fresh skim milk from which the casein had been separated according to the method of Van Slyke and Baker (8). The filtrate from the casein was boiled for 10 minutes in a steam-jacketed kettle and the coagulum washed several times with hot water and dried in the usual way with alcohol and ether. The material contained 15.44 per cent N and 2.76 per cent cystine, calculated on an ash- and moisture-free basis. The cystine was determined according to the Sullivan method.

100 gm. of the lactalbumin were suspended in 1.5 liters of 2 per cent  $\text{Na}_2\text{CO}_3$  solution and the mixture was heated in an oil bath with vigorous stirring. After all the protein was dispersed, which required about 25 minutes, the stirring was discontinued and the contents of the flask were boiled under a reflux for 45 minutes. The cooled solution was then poured into 1 liter of water in a large jar, and acetic acid was carefully added until no further precipitation occurred. The precipitate was collected on cheese-cloth, and washed several times with hot water. The separated material was hydrolyzed with boiling 20 per cent HCl for 48 hours. From the acid hydrolysate there was isolated 0.25 gm. of lanthionine by following the same procedure described for its isolation from the hydrolysate of the  $\text{Na}_2\text{CO}_3$ -treated wool.

*From Sodium Sulfide-Treated Wool*—100 gm. of wool were boiled for 1 hour with 2 per cent  $\text{Na}_2\text{S}$  solution. At the expiration of that time practically all of the wool was dispersed. After filtration the liquid was poured into 1 liter of water, and acetic acid was added until no further precipitation occurred. The curd-like precipitate was collected on cheese-cloth, washed, and hydrolyzed by boiling with HCl for 18 hours. The hydrolysate yielded 0.8 gm. of lanthionine.

*From Hair Treated with 0.1 N NaOH*—100 gm. of hair were boiled with 0.1 N NaOH for 1 hour. The treated hair was collected on cheese-cloth, washed with water, and hydrolyzed by boiling with HCl for 18 hours. 1 gm. of lanthionine was obtained from the hydrolysate.

The lanthionine isolated from the various sources described was identified in every instance by its crystal habit, N content, decomposition point,



microcrystallographic properties, and by the melting point of its dibenzoyl derivative (Table I).

Lanthionine is formed to a limited extent from proteins by the action of dilute alkali at much lower temperature than that used for the preparations described. A small quantity was obtained from wool that had stood in a 3 per cent solution of  $\text{Na}_2\text{S}$  for 1 week at room temperature. In

TABLE I  
*Characterization of Mesolanthionine Crystals Obtained from Various Sources*

Source	Crystal habit	N content	M. p. of dibenzoyl derivatives
		<i>per cent</i>	<i>°C.</i>
Human hair; boiled 1 hr. with 2% $\text{Na}_2\text{CO}_3$ solution	Six-sided plates, triangle-like appearance	13.39	206
Human hair; boiled 1 hr. with 0.1 N NaOH solution	" "	13.20	208
Chicken feathers; boiled 1 hr. with 2% $\text{Na}_2\text{CO}_3$ solution	" "	13.17	206
Wool; boiled 1 hr. with 2% $\text{Na}_2\text{CO}_3$ solution	" "	13.20	208
Lactalbumin; boiled 1 hr. with 2% $\text{Na}_2\text{CO}_3$ solution	" "	13.16	206

A sample of each of the dibenzoyl derivatives obtained from the sources indicated above when mixed with the corresponding derivative of previously identified lanthionine (3) showed no depression of the melting point.

Mr. G. L. Kecnan of the Microanalytical Division of the Food and Drug Administration, Federal Security Agency, kindly made the determinations of the refractive indices, which were  $n_D = 1.605$  and  $n_T = 1.647$  for each of the above mesolanthionine preparations.

another instance, about 0.1 gm. was isolated from 100 gm. of wool that had been suspended in a solution of  $\text{Na}_2\text{S}$  for 6 days at  $37^\circ$ .

#### SUMMARY

Lanthionine, a thio ether amino acid first isolated from  $\text{Na}_2\text{CO}_3$ -treated wool, has been similarly obtained from human hair (2.5 per cent), chicken feathers (0.25 per cent), and lactalbumin (0.25 per cent). It has also been isolated (1 per cent) from acid hydrolysates of hair that had been pretreated by being boiled for 1 hour with 0.1 N NaOH solution.

Dilute alkali reacts with wool even at relatively low temperatures. Lanthionine (0.1 gm.) was isolated from 100 gm. of wool that had been suspended in 2 per cent solution of  $\text{Na}_2\text{S}$  for 6 days at  $37^\circ$ .

It appears probable that lanthionine may be similarly obtained from most proteins which yield cystine on acid hydrolysis.

In addition to lanthionine there was obtained about an equal amount of another compound having properties similar to lanthionine, but more soluble and differing in crystal form.

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# ISOLATION OF *dl*-LANTHIONINE FROM VARIOUS ALKALI-TREATED PROTEINS

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It has been noted in previous papers (1, 2) that, in addition to the triangle-like, hexagonal crystals of mesolanthionine obtained from the acid hydrolysates of alkali-treated proteins, there was also obtained in approximately the same quantity another crystalline compound having the properties of a thio ether amino acid. This compound was optically inactive and had the same elementary composition as the mesolanthionine, but it was more soluble and differed in crystalline appearance. The fact that it crystallized in different forms, depending on the solvent used, led us at first to believe that we were dealing with more than one substance. Sometimes it separated in the form of needles, and at other times as small hexagonal plates, or as relatively large prisms. When crystallized from hot water, a mixture of these different types of crystals was obtained. However, the different types of crystals all had the same composition and decomposition point, and were optically inactive. This peculiar crystallizing property is in marked contrast to that of mesolanthionine, which crystallizes in the same characteristic form irrespective of the solvent used.

By working with the combined preparations of the more soluble isomer obtained from several lots of  $\text{Na}_2\text{CO}_3$ -treated human hair, we have been able to obtain it by recrystallization from  $\text{NH}_4\text{OH}$  entirely in the form of thin, elongated plates, usually hexagonal in habit, of decomposition point  $283\text{--}284^\circ$ . When recrystallized from hot water, the compound separates as small prisms and hexagonal plates. Microcrystallographic examination of this product showed it to possess the property of dimorphism.

The elementary composition of this product, the fact that it contains no disulfide S, and the fact that all of its N is present as amino N leave no alternative but that it is an isomer of mesolanthionine. Its similarity to that thio ether amino acid in chemical and physical properties and its method of preparation from hair indicate definitely that it is one of the stereoisomers. The stereoisomeric forms of lanthionine have been recently prepared by Brown and du Vigneaud (3). The isomer we have isolated from hair is optically inactive and agrees closely in its properties and crystalline form with those given for the synthetic *dl*-lanthionine. It seems, therefore, that this compound can be no other than the racemic

form. Final proof will rest on its resolution into the two optically active components.

#### EXPERIMENTAL

100 gm. of human hair were washed with cold water and, without being dried, were boiled for 1 hour with 2 per cent  $\text{Na}_2\text{CO}_3$  solution. The treated hair was collected on a double layer of cheese-cloth and suspended in 200 cc. of concentrated  $\text{HCl}$ . After the mixture was boiled for 18 hours, the hydrolysate was treated with norit, and the nearly colorless filtered solution was concentrated *in vacuo* to a thick sirup and dissolved in about 1.5 liters of absolute alcohol. Pyridine was then added until a distinct odor of the reagent persisted after the mixture was stirred for 20 to 30 seconds. The precipitate, separated by centrifugation, was suspended in 225 cc. of hot water. After being heated to boiling, the material was cooled and allowed to stand in a refrigerator overnight. The precipitate which settled (7 to 10 gm.) was filtered off and later combined with similar preparations obtained from ten to twelve other lots similarly prepared. The combined precipitates, amounting to about 100 gm., were suspended in 500 cc. of water to which had been added 100 cc. of concentrated  $\text{NH}_4\text{OH}$ . The mixture was heated to boiling and, while still hot, the undissolved residue was filtered off by suction, washed with 50 per cent alcohol, and finally with absolute alcohol. The product, designated as Fraction I, consisted chiefly of mesolanthionine. After treatment with a little norit the volume of the filtrate was gradually reduced by distillation *in vacuo* on a water bath. As the ammonia was being removed, several separations of crystalline material were successively removed. In the fractionation of this material we were guided by examination of the crystals under a microscope. The first fractions to separate were the typical triangle-like, hexagonal crystals of mesolanthionine. These were combined with Fraction I. Continued concentration of the filtrate yielded crystals of various types, needles, parallelograms, short hexagonal plates, and elongated rectangular prisms, which collectively were designated as Fraction II. When the volume of the solution had been reduced to about 25 cc., an equal volume of alcohol was added and the precipitate obtained was added to Fraction II.

Fraction I amounted to approximately 50 gm., and consisted chiefly of mesolanthionine. In order to remove a small quantity of the more soluble *dl*-lanthionine, Fraction I was heated to boiling in 200 cc. of water and filtered. (The filtrate was reserved for use later in recrystallizing Fraction II.) The solid residue was then dissolved in hot water with the aid of  $\text{NH}_4\text{OH}$ . Acetic acid was added to the clear hot solution with stirring until the reaction was only slightly alkaline. Most of the mesolan-

thionine separated as large crystals which were filtered off while the solution was still hot. After cooling, the filtrate was acidified with acetic acid and allowed to stand overnight in the refrigerator, whereby a few more gm. were obtained. The total yield of mesolanthionine was 50 gm.

Fraction II, containing the *dl*-lanthionine, was suspended in the filtrate from Fraction I, referred to above, and the mixture was heated to boiling.  $\text{NH}_4\text{OH}$  was added in small portions with stirring. After the material had dissolved, a small amount of  $\text{NaCN}$  was added in order to reduce some cystine present to the more soluble cysteine. The solution was then acidified with acetic acid and allowed to stand in the refrigerator for 48 hours. The crystalline product which separated was suspended in 400 cc. of hot water and small portions of  $\text{NH}_4\text{OH}$  were added until a clear solution was obtained. Acetic acid was then cautiously added until crystals began to appear. As the still somewhat alkaline solution cooled, the *dl*-lanthionine separated in the form of beautiful, elongated hexagonal plates. After the separation of crystals had largely ceased, the product was filtered off from the still warm solution. In this way 10 gm. of *dl*-lanthionine were obtained in the form of lustrous crystals. On standing overnight in the refrigerator, the filtrate yielded an additional fraction of crystals in the form of a mixture of prisms, needles, and short hexagonal plates. This fraction weighed about 30 gm. By redissolving this fraction in water made strongly alkaline with  $\text{NH}_4\text{OH}$  and then expelling the excess of  $\text{NH}_3$  by evaporation most of the material was recovered in the form of elongated hexagonal plates.

Analysis of the *dl*-lanthionine gave the following percentage composition, which is in fairly good agreement with that previously reported for mesolanthionine (1).

$\text{C}_6\text{H}_{12}\text{SN}_2\text{O}_4$ .	Calculated.	C 34.59, H 5.81, N 13.46, S 15.41
	Found. <i>dl</i> -Lanthionine.	" 34.54, " 5.66, " 13.45, " 15.60
	" Mesolanthionine.	" 34.67, " 5.99, " 13.48, " 15.17

All of the N was found to be present in the amino form.

*Properties of dl-Lanthionine*—The compound is rather sparingly soluble in water. The solubility is about 0.15 gm. per 100 cc. of water at  $25^\circ$ , compared with 0.022 gm. for mesolanthionine under the same conditions. It is insoluble in alcohol, ether, chloroform, and acetone. When dissolved in hot dilute  $\text{NH}_4\text{OH}$ , and the solution is nearly neutralized with acetic acid, the compound separates as lustrous, elongated hexagonal plates (Fig. 1). It crystallizes from water as a mixture of crystals having different forms (Fig. 2). It decomposes at  $283\text{--}284^\circ$ . The decomposition point was the same for both the elongated plates that separated from dilute  $\text{NH}_4\text{OH}$  and the mixed crystals of different forms that separated from

water. A mixture of the two preparations also showed the same decomposition point. A 5 per cent solution of the *dl*-lanthionine in 2 *N* NaOH in a 1 dm. tube, and also a 2 per cent solution in 10 per cent HCl, showed no optical activity. It is stable toward alkalis and forms a yellow crystalline phosphotungstate. The dibenzoyl derivative made from several different preparations of the lanthionine melted at 195–198°.<sup>1</sup>

We are indebted to Mr. G. L. Keenan of the Microanalytical Division of the Food and Drug Administration, Federal Security Agency, for the photomicrographs of the *dl*-lanthionine (Figs. 1 and 2), and for the following characterization of the crystals.

*"Note on the Dimorphic Forms of dl-Lanthionine"*—In connection with the micro-crystallographic examination of samples of *dl*-lanthionine crystallized from ammonia and water, respectively, it was observed that the habit of the crystals was markedly

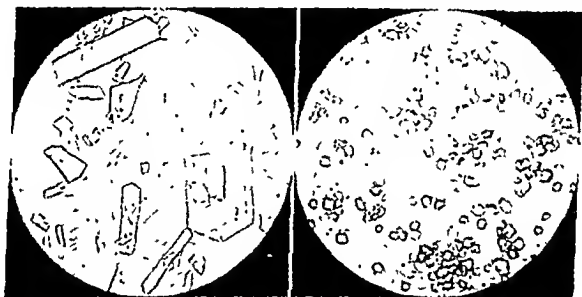


FIG. 1

FIG. 2

FIG. 1. *dl*-Lanthionine, crystallized from ammonia.

FIG. 2. *dl*-Lanthionine crystallized from water.

different, also the principal optical-crystallographic properties. These samples as prepared were represented as being analytically identical, optically inactive, and containing no solvent of crystallization.

"The material crystallized from ammonia forms elongated, rod-shaped plates, usually hexagonal in habit (Fig. 1). When examined microscopically with crossed nicols (parallel polarized light), the sign of elongation is plus and the extinction is parallel. Intermediate values of the refractive indices are usually shown on the substance and especially  $n = 1.660$  to  $1.665$  crosswise on elongated plates. The maximum index is greater than  $1.695$  but less than that for methylene iodide ( $1.733$ ). No interference figures could be observed in convergent polarized light (crossed nicols).

"The material crystallized from water, on the other hand, consisted of numerous small prisms and hexagonal plates (Fig. 2). In the absence of interference figures, the statistical method for the determination of the refractive indices was used, the

<sup>1</sup> The melting point of our dibenzoyl preparations was uniformly somewhat higher than that given by Brown and du Vigneaud (3). They give 183–184°.

following minimum and maximum values being obtained:  $n_{\alpha} = 1.567$ ,  $n_{\gamma} = 1.677$ , both  $\pm 0.003$ .

"It is apparent that these are two different forms of the same substance and it is interesting to observe that the form crystallized from water can be readily changed over to the other form by crystallizing from ammonia on a microscope slide."

#### SUMMARY

An isomeric thio ether amino acid, previously observed accompanying mesolanthionine in approximately the same proportion in the hydrolysates of certain alkali-treated proteins, has been isolated from  $\text{Na}_2\text{CO}_3$ -treated human hair. Because it is optically inactive, but differs from mesolanthionine in solubility and crystalline form, and because it agrees closely in its properties and crystalline form with those given for the synthetic *dl*-lanthionine, it seems that this compound can be no other than *dl*-lanthionine. Although sparingly soluble in water, it is approximately 7 times as soluble as mesolanthionine. It possesses the property of dimorphism. It crystallizes from dilute ammonia as lustrous, elongated hexagonal plates, decomposing at  $283\text{--}284^\circ$ , but from water it separates as crystals belonging to a different crystal system and having several different crystal habits.

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# THE AMOUNT AND DISTRIBUTION OF CYTOCHROME OXIDASE IN BULL SPERMATOOZOA\*

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The studies of Lardy and Phillips (1) have shown that bull spermatozoa contain succinic dehydrogenase, a catalyst intimately connected with the cytochrome system in tissues (2). The earlier work of Iwanow (3) had shown that the greater part of the respiration of mammalian spermatozoa was cyanide-sensitive, suggesting the probable presence of the cytochrome system. There seems to be general agreement that cytochrome oxidase, the enzyme which, as the name implies, catalyzes the oxidation of reduced cytochrome *c*, is the chief respiratory factor affected by cyanide (4). In view of these facts, even though little or no cytochrome can be detected in bull spermatozoa by spectroscopic examination (1),<sup>1</sup> estimations have been made of the cytochrome oxidase. The oxidation of secondary substances such as *p*-phenylenediamine and hydroquinone in the presence of excess cytochrome *c* has been used as a measure of the cytochrome oxidase. The method recently described by Stotz (4) has been followed in most details.<sup>2</sup> Earlier chemical studies (6)<sup>1</sup> with the parts, designated heads, midpieces, and tails, of disintegrated spermatozoa had revealed striking differences in chemical composition. The distribution of oxidase in these parts has therefore been studied also; differences have been found which may be correlated with differences in function of the parts.

## EXPERIMENTAL

*Preparation of Materials*—The spermatozoa were washed with water, as described previously (7), from the excised cauda epididymidis of six to ten fresh bull testicles, obtained from the abattoir within 2 hours after

\* This work has been aided by a grant from the National Committee on Maternal Health, Inc

<sup>1</sup> Zittle, C. A., and Zitin, B., *J. Biol. Chem.*, **144**, 105 (1942).

<sup>2</sup> The estimation of cytochrome oxidase described by Stotz (4) is based on the oxidation of hydroquinone. Earlier studies (5) had shown that the use of *p*-phenylenediamine was a less accurate measure of cytochrome oxidase in the presence of cytochrome *b* which can oxidize *p*-phenylenediamine independently of oxidase. Our measurements have been based largely on the use of *p*-phenylenediamine. The interpretation of the oxidation obtained with this substrate without excess cytochrome *c* will have the limitation mentioned above. However, the increase in oxidation obtained with excess cytochrome *c* is clearly due to the presence of cytochrome oxidase.

inactivated at 38° by hydroquinone but not at 20°, we performed an experiment at the latter temperature with ground spermatozoa. In this experiment hydroquinone, *p*-phenylenediamine, and sodium succinate were used as substrates in parallel. The c.mm. of O<sub>2</sub> per mg. per hour were 4.8, 7.7, and 8.3, respectively, with excess cytochrome *c*. With inactivated spermatozoa 3.1 c.mm. of O<sub>2</sub> per mg. per hour were taken up with hydroquinone but none with the other substrates. The O<sub>2</sub> taken up by oxidase activity with hydroquinone again was considerably smaller than that with the other substrates. No explanation can be given at present for these unusual results with hydroquinone. It may be that in these preparations the enzyme is largely inactivated by hydroquinone even at 20°. However, it seems to be more stable in the preparations obtained by sonic treatment.

TABLE I

*O<sub>2</sub> Consumption of Sonically Disintegrated Spermatozoa with p-Phenylenediamine As Substrate at 37°*

The inactive preparations of spermatozoa consumed no O<sub>2</sub> with *p*-phenylenediamine.

Test system	Disintegrated spermatozoa		Heads		Midpieces		Tails	
		No. of ex- peri- ments		No. of ex- peri- ments		No. of ex- peri- ments		No. of ex- peri- ments
	c.mm. per mg. per hr.		c.mm. per mg. per hr.		c.mm. per mg. per hr.		c.mm. per mg. per hr.	
Excess cyto- chrome <i>c</i>	25.0 (21.0-29.6)	6	1.2 (0.8-1.5)	5	14.4 (6.2-18.7)	5	29.1 (26.1-31.8)	5
No added cyto- chrome <i>c</i>	5.1 (4.7-5.5)	2	0.2 (0.1-0.2)	3	3.8 (2.1-5.9)	3	12.0 (11.3-12.8)	3

Results of experiments performed with heads, tails, and midpieces of spermatozoa are given in Table I where the O<sub>2</sub> taken up with and without added cytochrome *c* is tabulated.<sup>5</sup> Large differences were obtained with the different parts. The fraction designated tails is, as previously described (6), difficult to sediment in the centrifuge. In addition to the particles by which this fraction was characterized microscopically, some of the material forms an opalescent, colloidal solution. Since this might be regarded as lost from the other parts of the spermatozoa, the particles and the colloidal solution of the tail fraction were separated by centrifugation and tested separately (the data shown in Table I are for the total tail

<sup>5</sup> The amount of heads used in these tests was larger than that of the midpieces and tails in order to have a measurable O<sub>2</sub> uptake. In a typical experiment 18.8, 1.3, and 2.9 mg. of heads, midpieces, and tails, respectively, were used.

material). In the two experiments in which this was done the two fractions gave essentially the same results. Hence the high oxidase content of the tails as such is confirmed. Since microscopically the heads and midpieces do not show any alteration during the sonic treatment and the visible particles of the tail show a gradation in size, we believe that the colloiddally dispersed material remaining in the supernatant fluid after centrifugation also represents tail material.

#### DISCUSSION

The data obtained confirm the presence of the cytochrome system in bull spermatozoa. The cytochrome oxidase content per mg. (2.5 units<sup>6</sup>) is about the same as was found by Stotz (4) in rat skeletal muscle (2.3 units), and rat liver, spleen, lung, and testis (1.1 to 1.7 units), but much less than the amounts found in rat heart (9.7 units). Krahle *et al.* (11) found about 1 unit per mg. of tissue at 20° in *Arbacia* eggs. With this material no O<sub>2</sub> was consumed unless cytochrome *c* was added. Presumably the O<sub>2</sub> uptake obtained with spermatozoa with no added cytochrome *c* is due to cytochrome *c* originally present in the preparation of spermatozoa, although with *p*-phenylenediamine some may be due to cytochrome *b*.<sup>2</sup>

The large differences in the amounts of oxidase and cytochrome in the heads, midpieces, and tails are probably correlated with their different functions. The head is probably a metabolically rather inert carrier of the chromosomes (it contains over 40 per cent of nucleic acid (6)), whereas association of the cytochrome system with the midpieces and tails, parts of an organ of locomotion, would not be surprising. Recently the association of the cytochrome system with the most active tissues has been emphasized again (12).

#### SUMMARY

The O<sub>2</sub> consumed by spermatozoa, disintegrated by grinding or by sonic treatment, with *p*-phenylenediamine as the substrate in the presence of excess cytochrome *c* at 37° has been used as a measure of cytochrome oxidase. An average O<sub>2</sub> consumption of 25 c.mm. per mg. per hour was found with sonically treated spermatozoa. The O<sub>2</sub> consumption by the parts of the spermatozoa in the same test system was heads 1.2, midpieces 14.4, and tails 29.1 c.mm. per mg. per hour. These differences are discussed in relation to the function of the different parts.

Similar experiments were performed with hydroquinone and sodium succinate as the substrates. The results with sodium succinate were of the

<sup>6</sup> 1 unit of oxidase has been defined (4) as the amount which produced an increase in O<sub>2</sub> consumption of 10 c.mm. per hour over the autoxidation rates under the specified conditions.

*Spermatozoal Fractions Obtained after Sonic Vibration*<sup>2</sup>—The treatment of spermatozoa by sonic vibration and subsequent separation by differential centrifugation into three parts, designated heads, midpieces, and tails, has been described in detail (1).

*Spermatozoal Fractions Obtained after Use of Homogenizer*<sup>3</sup>—A small hand-operated homogenizer was used to break up the spermatozoa in water suspension by repeated homogenization (ten to twenty times). Differential centrifugation was used to separate the broken parts. A fraction composed of pure heads was obtained by this means. However, the break between the midpiece and tail did not occur at a morphological division as with the previous method. The break seemed to occur at random in the anterior portion of the tail. This was observed in microscopic examination of the fractions and confirmed by weight ratios and P contents at variance with the data obtained with the fractions obtained after sonic vibration (1).

*Analytical Methods for Total Iron*—The ashing procedure is an important part of the determination of Fe in biological material. A detailed survey of various ashing methods and the factors involved are given by Jackson (9). A comparison of ashing (10) and of digestion (11) methods for Fe in spermatozoa led us to adopt the latter procedure. 75 to 400 mg. of spermatozoa were digested in a 100 ml. Kjeldahl flask with 2 ml. of concentrated  $H_2SO_4$ , 0.5 to 1.0 ml. of 70 per cent  $HClO_4$ , and one glass bead (acid-extracted). The mixture was heated until all fumes of  $HClO_4$  were driven off and the solution was clear and colorless. The flask was heated for 10 minutes more and then allowed to cool. The following colorimetric methods were then used, any one being satisfactory.

*Method 1; Thiocyanate*—The method of Kennedy (11) was used with slight modifications according to the recommendations of McFarlane (12). Details of the method follow. To the digested liquid in the flask were added a few ml. of  $H_2O$  followed by 2 drops of concentrated  $HNO_3$  to oxidize the Fe. The mixture was allowed to cool and then was washed into a 15 × 195 mm. test-tube. 4 ml. of a 20 per cent solution of KCNS and 5 ml. of isoamyl alcohol were added. The mixture was shaken vigorously for about 30 seconds and allowed to separate into two layers. The colored alcohol phase was then transferred to colorimeter tubes and the color read within 30 minutes. If a slight emulsion formed, giving a cloudy solution, as was sometimes the case, the mixture was centrifuged for a few minutes until clear.

\* The sonic disintegration of spermatozoa was performed at the Johnson Foundation for Medical Physics through the kindness of Dr. L. A. Chambers.

\* The use of a high pressure homogenizer was suggested by Dr. L. A. Chambers. Further investigation showed that the small hand model would break the spermatozoa equally well.

*Method 2; Thioglycolic Acid*—The details of this method were based on the procedures of Leavell and Ellis (13), Lyons (14), and Tompsett (15). The reagents, prepared according to Tompsett, were added directly to the flask after digestion of the sample. The purple color which developed (pH 8 to 10) was read in the colorimeter tubes. The solutions were shaken while exposed to air before comparison to obtain the maximum intensity of color.

*Method 3; o-Phenanthroline*—The method of Saywell and Cunningham (16), as adapted to biological materials by Hummel and Willard (17), was used. Hydroquinone was used as the reducing agent. The reagents were added directly to the digested sample, mixed, and allowed to stand for 1 hour to insure complete conversion to the ferrous complex, before being transferred to the colorimeter tubes for reading.

*Standard and Colorimetry*—A stock standard Fe solution containing 0.1 mg. of ferric Fe per ml. of solution was prepared according to the method of Kennedy (11) from Merck's reagent quality Fe wire. This solution was diluted as required. The Fe concentrations in the standards and unknown samples were kept as close as possible. All readings were corrected for the reagent blank before calculations were made. A Klett-Summerson photoelectric colorimeter with a green filter No. 54 was used for reading the colors. For Method 1, 40 to 200 mg. of spermatozoa gave sufficient color for a determination; for Methods 2 and 3, at least 300 and 400 mg. of spermatozoa respectively were used.

*Analytical Method for Non-Hemin Iron*—The non-hemin Fe was determined by the method of Brückmann and Zondek (18, 6) by extraction with pyrophosphate and trichloroacetic acid at 100° of samples ground with glass powder. The Fe in the extracts was determined by one of the colorimetric methods. Three extractions were found to be sufficient for each sample; the Fe was determined for each extraction and blanks and standards were run simultaneously. The Fe could be determined directly in the extracts with colorimetric Methods 2 and 3 but it was found to be more desirable to digest the extracts before the color was developed, since interference due to pigments, opalescence, etc., was avoided. Pyrophosphate interferes with the direct use of Method 1. Hydroquinone could not be used in the presence of pyrophosphate but Method 3 could be used directly when a pinch of powdered sodium hydrosulfite was substituted for the hydroquinone.

Since there was some doubt (5) of the complete solubility of the Fe in the presence of trichloroacetic acid and pyrophosphate, we repeated the experiments of Brückmann and Zondek (6) in which the amounts of these reagents added to standard Fe solutions were varied. The mixtures were heated and centrifuged and the Fe determined. The data shown in Table I confirm the statement of Brückmann and Zondek (6) that Fe-pyrophos-

phate is soluble under these conditions. However, we observed that when the extracts stood overnight before the *o*-phenanthroline reagent was added, less color was subsequently developed. Apparently under these conditions a complex between Fe and pyrophosphate is formed slowly which is not dissociated with the reagent used. No precipitate was observed.

*Analytical Method for Hemin Iron (Direct Method)*—The direct determination of the hemin Fe content was attempted by means of the acidified methyl alcohol extraction method described by Yabusoe (19). This method gave negative results with our material, probably in part owing to the very small quantity of hemin Fe present as deduced from the determination of total and non-hemin Fe. Further, the spermatozoa are ex-

TABLE I  
Summary of Iron Recovered in Presence of Pyrophosphate

10 ml. CCl <sub>3</sub> COOH, concentration	Saturated Na <sub>2</sub> P <sub>2</sub> O <sub>7</sub>	0.0500 mg. iron	Iron recovered by different methods					
			Thiocyanate		Thioglycolic acid		<i>o</i> -Phenanthroline	
per cent	ml.		mg.	per cent	mg.	per cent	mg.	per cent
0	2.0	• Ferric			0.0489	98.0	0.0508	101.6
0	5.0	"			0.0496	99.4	0.0512	102.1
2.5	2.0	"	0.0500	100.0	0.0487	97.5		
2.5	5.0	"	0.0505	101.0	0.0504	100.5		
10.0	2.0	"	0.0500	100.0	0.0507	101.3	0.0513	102.6
10.0	5.0	"	0.0506	101.1	0.0504	100.6	0.0505	101.0
10.0	5.0	"			0.0511*	102.2*	0.0490*	98.1*
10.0	5.0	Ferrous			0.0517*	103.5*	0.0503*	100.4*

The amount of Fe in the reagents was determined and found to be less than 1 per cent of the amount added for the experiment.

\* Samples not ashed; all others were ashed and determined immediately after extraction.

tremely difficult to disintegrate and the non-aqueous extractant used may not have given the necessary dissolution of the spermatozoa for the specific solvent action of methyl alcohol for hemin compounds to be exerted.

*Spectroscopic Examination for Cytochrome c*—A spectroscopic examination of spermatozoa was performed with a Leitz hand spectroscope with an attached wave-length scale, according to the method of Ball and Meyerhof (20) who demonstrated cytochrome *c* in the spermatozoa of *Arbacia punctulata*. We examined dry and fresh samples of bull spermatozoa, both sonically treated and ground with glass, adding powdered sodium hydrosulfite as a reducing agent to water and trichloroacetic acid suspensions of spermatozoa. No absorption bands at all were discernible with our equipment.

### Results

The amount of total Fe found in bull spermatozoa with the three methods used is summarized in Table II. The three reagents gave identical results within the limits of error of the methods, which was  $\pm 5.0$  per cent. The range of values shown was obtained with different lots of spermatozoa which in every case represented a pool of the spermatozoa from several testicles.

The total Fe in the spermatozoal parts obtained by sonic disintegration and homogenization was determined by the thiocyanate method, since smaller amounts of material were needed. In every case a preparation of whole spermatozoa was determined simultaneously for comparison. The data for whole spermatozoa are included in Table II. The data for the various spermatozoal parts showed considerable variability. This was eventually traced to contamination with Fe by the methods used to break

TABLE II  
*Total Iron Content of Bull Spermatozoa*

Method used	No. of samples	No. of determinations	Iron, average	Range
			<i>per cent</i>	<i>per cent</i>
Thiocyanate.....	32	73	0.00713	0.00610-0.00860
Thioglycolic acid.....	5	11	0.00729	0.00660-0.00891
<i>o</i> -Phenanthroline.....	6	6	0.00689	0.00664-0.00716
Average.....			0.00710	

up the spermatozoa. The sonic vibrations were imparted to the spermatozoal suspension by a nickel tube which was found to contain a small amount of Fe. The tube underwent slight disintegration owing to the intense cavitation and in some cases small particles separated. These particles were observed in the bottom of centrifuged samples and in several cases were shown to contribute some Fe. Data obtained when the Fe-containing particles were removed and consideration of the minimum values for Fe found indicate the following approximate Fe contents of the parts, 0.004, 0.02, and 0.01 per cent, respectively, for heads, midpieces, and tails. The Fe content of the fractions obtained by homogenization was usually so high that again contamination could be suspected. We believe this contamination also came from the apparatus used, since the water used in preparing suspensions of spermatozoa for such treatment and the subsequent separation was Fe-free.

The non-hemin Fe was determined in bull spermatozoa and, for comparison,



in bull testicle.<sup>4</sup> Brückmann and Zondek (6) have previously studied human testicle. The data obtained by us are given in Table III. The values found for bull testicle are of the same order as were found by Brückmann and Zondek (6) in testicular material. The data obtained

TABLE III  
*Non-Hemin Iron Content of Bull Spermatozoa and Testicles*

	Sample No	Iron by 1st extraction	Iron by 2nd extraction	Iron by 3rd extraction	Total non hemin iron	Total iron	Hemin iron (by difference)
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Spermatozoa	1	0.00264	0.00110	0.00012	0.0039	0.0071	0.0032
	2	0.00234	0.00180	0.00012	0.0043	0.0071	0.0028
	3	0.00284	0.00178	0.00000	0.0046	0.0071	0.0025
	4	0.00342	0.00098	0.00012	0.0045	0.0071	0.0026
Average					0.0043	0.0071	0.0028
Testicles	1	0.0108	0.0015	0.0000	0.0123	0.0131	0.0008
	2	0.00968	0.0017	0.0000	0.0114	0.0131	0.0017
Average					0.0119	0.0131	0.0013

with bull spermatozoa show that 60 per cent of the total Fe is non-hemin Fe. The remainder presumably is hemin Fe.

#### DISCUSSION

The amount of Fe that we have found in bull spermatozoa is not high and is of the same magnitude as has been found in other tissues. Rat testes and muscle, for example, contain a mean of 0.0149 and 0.0079 per cent, respectively (21). The approximate values which we obtained for the distribution of Fe suggest that the midpieces and tails are considerably richer in Fe than the heads. Other, indirect, evidence favors such a distribution for the hemin Fe at least. The dried lipid-free heads are pure white in color, whereas the midpieces and tails are a light buff color. The colored hemin compounds may cause this color. Further, the cytochrome oxidase and cytochrome *c* of bull spermatozoa are concentrated largely in the midpieces and tails (22). The oxidase is probably an Fe-containing enzyme (23) and the cytochromes are hemin-protein compounds (24).

The nature of the non-hemin Fe of spermatozoa is not known. The heads and midpieces contain large amounts of nucleic acid (1) and perhaps complexes are formed between the Fe and nucleic acid of the type described by Fischer and Hultzscl (4).

<sup>4</sup> This was prepared by removing the highly vascular capsule of the testicle, grinding the inner portion, and drying in a vacuum from the frozen state

The amount of hemin Fe in spermatozoa, deduced from the total and non-hemin Fe, although apparently small, nevertheless in terms of Fe-containing enzymes would be relatively large. Cytochrome *c*, for example, contains only 0.43 per cent of Fe (25). Our inability to demonstrate cytochrome *c* spectroscopically confirms the experience of Lardy and Phillips (26) with the same material and the results of MacLeod (27) with human spermatozoa. This method is limited by the difficulty of getting a satisfactory dispersion of the spermatozoa. The cytochrome system is present in bull spermatozoa (22).

#### SUMMARY

The total Fe content of dried lipid-free bull spermatozoa was determined colorimetrically with the thiocyanate, thioglycolic acid, and *o*-phenanthroline reagents. A mean value of 0.0071 per cent was found. The Fe content of parts of the spermatozoa prepared from spermatozoa broken by sonic vibrations and homogenization was determined also but the accuracy was impaired by contamination from the apparatus used. However, definitely more Fe was present in the midpieces and tails of the spermatozoa than in the heads. Cytochrome *c* could not be detected spectroscopically.

The non-hemin Fe of bull spermatozoa was estimated to be 60 per cent of the total Fe. Similar determinations were carried out on bull testicles. Experiments were performed which showed that the method used, extraction with hot pyrophosphate, was not limited by the formation of an insoluble Fe-pyrophosphate compound.

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meal (S. B. Penick) by the use of the method of Bredereck (6). Since an exact characterization of the enzymes that are present in these preparations is not possible at the present time, its action throughout this publication is conveniently referred to as "non-specific phosphatase."

Duplicate weighed samples of ribonucleic acid, previously dried to constant weight *in vacuo* over sulfuric acid, were transferred quantitatively into 200 cc. volumetric flasks with the aid of a measured quantity of a solution of sodium hydroxide of known concentration. Exactly sufficient

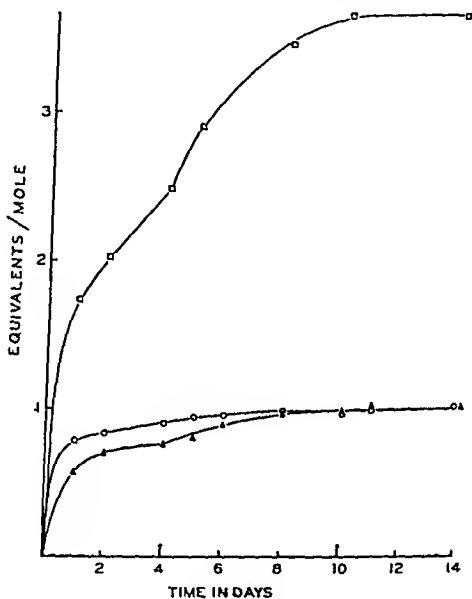


FIG. 1. The hydrolysis of ribonucleic acid by non-specific phosphatase after previous treatment of the ribonucleic acid with ribonuclease. □ inorganic phosphate, ○ guanosine, △ adenosine. Extra additions of non-specific phosphatase are indicated by the arrows at the 4th and 10th days.

sodium hydroxide was used to bring the pH of the solutions to 7.0, as calculated from titration data. Ribonuclease was added to one of the duplicate solutions, which was then incubated at 37° for 24 hours to insure complete ribonuclease action. Finally, 100 cc. of 0.2 M acetate buffer (pH 5.1) and 50 cc. of 1 per cent non-specific phosphatase solution were added to each solution. The volume was adjusted to 200 cc. by the use of distilled water. A few drops of toluene were added and the solutions were incubated at 37°. Every 24 hours, aliquots were withdrawn

and analyzed for inorganic phosphate, total soluble purine nucleoside nitrogen, and soluble guanosine nitrogen.

Deproteinization of the solution for the determination of inorganic phosphate by the method of Fiske and Subbarow (7) was effected by the addition of 6 volumes of a 5 N solution of sulfuric acid, 12.5 volumes of a 2.5 per cent solution of  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$  in 2.5 N sulfuric acid. The deproteinization was carried out in a 100 cc. volumetric flask; so that the final volume before filtration from the flocculated protein was adjusted to

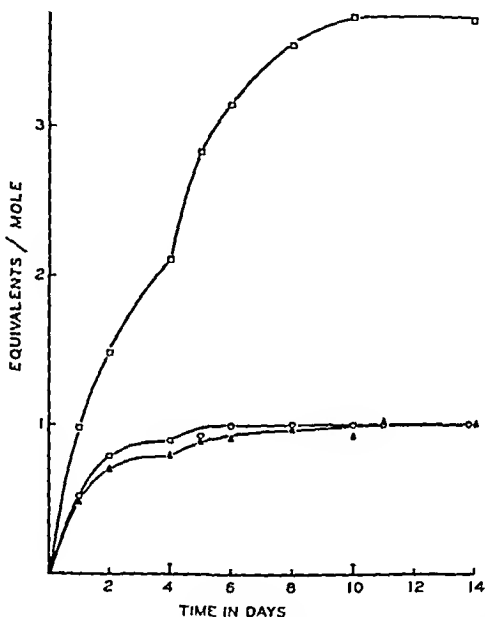


FIG. 2. The hydrolysis of ribonucleic acid by non-specific phosphatase. □ inorganic phosphate, ○ guanosine, Δ adenosine. Extra additions of non-specific phosphatase are indicated by the arrows at the 4th and 10th days.

100 cc. 40 cc. of the filtrate were taken for analysis. This method was resorted to in order to avoid the development of turbidity due to the presence of soluble nucleotides. Ribonucleic acid as well as the proteins present in the phosphatase preparations when precipitated from solutions was found to absorb inorganic phosphate. This was corrected in every case by parallel analyses on solutions of inorganic phosphate of known concentration and of enzyme preparation or of ribonucleic acid.

The deproteinization of samples for the determination of total soluble

purine nucleoside nitrogen and of guanosine nitrogen was performed by the use of a 1.25 per cent solution of uranyl acetate in 10 per cent trichloroacetic acid as described by MacFadyen (8). After the removal of protein and ribonucleic acid, the nucleotides together with inorganic phosphate and excess uranyl ion were removed from the solution by the method of Kerr (9). Nucleosides are present in the resultant solution. In order to reduce the absorption of nucleosides to a minimum and to obtain check results the precipitated nucleotides and uranyl phosphate must be redissolved with 1 cc. of 1  $N$   $H_2SO_4$  and reprecipitated three times. The hydrolysis of the purine nucleosides and determination of total purine nitrogen were carried out according to the method of Kerr (9). Guanosine was determined by applying the Hitchings (10) method for guanine to an aliquot of the foregoing nucleoside hydrolysate. The content of adenosine is calculated by the difference between the contents of total purine nucleoside and guanosine. A Fisher photoelectric colorimeter was used in the colorimetric procedures.

The data for one of six similarly planned experiments in which different samples of non-specific phosphatase as well as ribonucleic acid were used are presented graphically in Figs. 1 and 2.

#### DISCUSSION

An examination of the data shows that the action of non-specific phosphatase preparations, as measured by the inorganic phosphate as well as the purine nucleosides that are liberated from ribonucleic acid, is more rapid when ribonucleic acid is first subjected to the action of ribonuclease. Thus, for example, in the sample of ribonucleic acid subjected to the action of ribonuclease and subsequently to the action of non-specific phosphatase, 0.78 equivalent of guanosine, 0.57 equivalent of adenosine, and 1.73 equivalents of inorganic phosphate are liberated in the first 24 hours of enzymic action. These values are in marked contrast to 0.52 equivalent of guanosine, 0.48 equivalent of adenosine, and 0.97 equivalent of inorganic phosphate that are liberated in 24 hours from the sample of ribonucleic acid which had been treated only with the non-specific phosphatase preparation. On the basis of the inorganic phosphate that is liberated in the parallel experiments, the hydrolytic action of non-specific phosphatase is 75 per cent greater when ribonucleic acid is previously subjected to the action of ribonuclease. Five other experiments, conducted similarly to that shown graphically, but in which different preparations of phosphatase as well as ribonucleic acid were used, gave values which ranged from 50 to 150 per cent higher for the hydrolytic action of non-specific phosphatase on ribonucleic acid that had been previously treated with ribonuclease in comparison to the action on untreated ribonucleic

acid. As the time of hydrolysis is extended, the data for the parallel experiments become similar between the 4th and 6th days. The indication is that the hydrolytic action has eventually become analogous.

The significance of the foregoing data becomes more apparent if they are examined in the light of the following question. Can native ribonucleic acid be hydrolyzed enzymically to its components by the use of non-specific phosphatase without previous action on the molecule by the enzyme ribonuclease? That the non-specific phosphatase preparations employed in this investigation contained small amounts of ribonuclease was ascertained by treatment of the preparations with heat and testing qualitatively in the manner described by Dubos and Thompson (11). The amounts of ribonuclease that are present are small, and hence escape detection by the Dubos and Thompson procedure unless the period that is allowed for its action is extended to 24 hours. With this fact established, it is apparent that the differences that are noted in the parallel experiments are not attributable to the effect of different enzymes, but rather to the relative amounts of ribonuclease that are present. In one case, sufficient crystalline ribonuclease was added to insure complete reaction before the addition of the non-specific phosphatase preparation; in the parallel case, ribonuclease action proceeded simultaneously with that of the non-specific phosphatase. It thus seems reasonable to conclude that the enzymic hydrolysis of ribonucleic acid to its components requires the action of ribonuclease prior to the action of non-specific phosphatase.

Regarding the relative positions of the mononucleosides in the tetranucleotide unit of ribonucleic acid, reference to the data shows that in the early stages of the parallel hydrolyses guanosine is liberated at a faster rate than adenosine. This is especially marked in the sample that received previous treatment with ribonuclease. The equivalents of inorganic phosphate that are liberated in the sample subjected only to the action of the non-specific phosphatase are, within the experimental error, equal to the equivalents of purine nucleosides that are liberated in the first 48 hours. This is definite proof that no one of the pyrimidine components has been hydrolyzed. The hydrolysis of the pyrimidine components in quantity occurs only as the reaction is extended to the point where the hydrolysis of the purine components approaches the maximum. In the sample that received treatment with ribonuclease and subsequently with non-specific phosphatase, the equivalents of inorganic phosphate that are liberated are greater than the equivalents of purine nucleosides for all periods of the hydrolysis. Thus, as would be expected in the more rapid reaction, a small percentage of the pyrimidine components is hydrolyzed in the early stages and increases as the hydrolysis of the purine components approaches the maximum.

This information seemingly suggests that (1) the enzymic degradation of the tetranucleotide unit of ribonucleic acid proceeds by way of a successive series of attacks upon the inorganic phosphate and nucleoside linkages that are at the ends of the molecule; (2) guanylic and adenylic acids occupy positions in the tetranucleotide unit such that they are subject to enzymic degradation before the pyrimidine-containing nucleotides. If both ends of the tetranucleotide unit are subject to like enzymic action, guanylic and adenylic acid *may occupy the two outer positions in the molecule*. If only one position of the tetranucleotide unit is found to be the position of attack, guanylic and adenylic acids then occupy adjacent positions in the molecule, with the outer position occupied by guanylic acid. A recent report of Bredereek, Berger, and Richter (12) that is available at the present time only in the form of an abstract may also be interpreted to allocate the positions of the nucleotides in either (a) the two outer positions or (b) adjacent positions in the tetranucleotide structure.

In conclusion, it has been shown that the action of ribonuclease upon native ribonucleic acid hydrolyzes a linkage which permits a more rapid enzymic hydrolysis of the resultant molecule to take place as a result of non-specific phosphatase action. The advent of methods for the preparation of pure monophosphatase and diphosphatase may bring to light a total dependence of the action of these enzymes on native ribonucleic acid and upon a previous depolymerization of the molecule by ribonuclease. Also, it has been shown that the relative positions of the nucleotides in the tetranucleotide structure are not the generally accepted ones assigned by Levene and Simms (3). The nucleotide in the outer position in the tetranucleotide chain in which a secondary hydroxyl of a phosphate group becomes free after ribonuclease action is that of guanylic acid. Adenylic acid may then be allocated to one of two possible positions, (1) adjacent to guanylic acid or (2) *in the outer position of the tetranucleotide chain furthest removed from guanylic acid*.

#### SUMMARY

The enzymic action of non-specific phosphatase preparations on ribonucleic acid has been compared to that in which ribonucleic acid has been subjected previously to the action of ribonuclease.

On the basis of the inorganic phosphate that is liberated, the hydrolytic action of non-specific phosphatase is 50 to 150 per cent greater on ribonucleic acid that had been treated previously with ribonuclease in comparison to the action on untreated ribonucleic acid.

The action of ribonuclease is correlated with that of non-specific phosphatase and a tentative procedure whereby the stepwise hydrolysis of

ribonucleic acid may be accomplished by the action of these enzymes is offered.

The data for the rates of liberation of the purine nucleosides from ribonucleic acid permit the allocation of these nucleosides into certain specific positions in the ribonucleic acid structure.

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## AMINO ACID STUDIES

### I. PLASMA AMINO ACID RETENTION IN THE HYPOPROTEINEMIC DOG AS EVIDENCE OF IMPAIRED LIVER FUNCTION\*

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In the past investigators have attempted to evaluate liver function by means of amino acid loading curves in patients and experimental animals. Kitamura (1) demonstrated a retention<sup>1</sup> of plasma amino acid following intravenous injection of single amino acids in rabbits with livers injured by chloroform and other chemical poisons. Extirpation experiments (2, 3) in the dog had demonstrated that between 80 and 90 per cent of the liver must be removed before a reduction in deamination and urea synthesis can be demonstrated. In patients with liver disease, Weicker (4) and Kirk (5), although able to demonstrate slight plasma retention following the ingestion of amino acids in some patients, interpreted their results as a failure to prove a reduction in deamination. Horejsi *et al.* (6) felt that little information in regard to liver function could be gained from intravenous injection of glycine into patients unless very severe liver destruction was present. Morphological changes in the liver in some edematous dogs fed a low protein diet have been noted in this laboratory (7, 8).<sup>2</sup> Elman and Heifetz (9) elaborated on these findings and studied liver function in dogs rendered hypoproteinemic in the same manner. They found that progressive impairment in liver function and morphological injury paralleled the decline in serum albumin. With the advent of the ninhydrin method of Van Slyke, Dillon, MacFadyen, and Hamilton (10) we were led to investigate, in normal and in hypoproteinemic dogs, certain aspects of the amino acid metabolism in search of possible correlation with liver injury.

#### *Procedure*

In the experiments reported here, three young mongrel female dogs served as their own controls before hypoproteinemia was induced. They

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<sup>1</sup> Retention will be used throughout the paper to connote increased plasma values of amino acid N.

<sup>2</sup> Weech, A. A., and Goettsch, E., unpublished data.

readily accepted a balanced diet, previously described (8), to which sufficient casein was added to fulfil the normal protein requirement. Plasma amino acid curves were obtained after the intravenous injection of 20 cc. of a 10 per cent solution of casein hydrolysate<sup>3</sup> diluted with an equal amount of 5 per cent glucose in saline. Over 2 years time three lots of casein hydrolysate were obtained which varied somewhat in amino acid N content. However, the same lot of material was used throughout the experiments on a single animal. The average amino acid N content was 6.8 mg. per cc. of a 10 per cent solution of the dried material. The average total N content was 13.0 mg., the difference being probably largely polypeptide N due to incomplete enzymatic hydrolysis. Since the dogs weighed 10 to 11 kilos, about 12 mg. of amino acid N per kilo were administered. The dogs were fasted about 18 hours before the injection, which required only 1 minute. Blood for the determination of amino acids, proteins, urea, and hematocrit was removed before the injection and at intervals thereafter; namely, 5 minutes, 15 minutes, 30 minutes, 1½ hours, and 3 hours. In order to determine the speed of the metabolism of casein hydrolysate the urine was collected so that a control period was obtained before the injection and three periods thereafter. The urine was collected by catheter and analyzed for urea, ammonia, total nitrogen, and amino acids, and the pH was determined by the glass electrode. From the data simultaneous urea and amino acid clearances were calculated.

Then the casein was removed from the diet and similar studies were made at intervals of 1 week or longer as hypoproteinemia progressed, until edema appeared. By removal of the casein only one dietary variable was introduced into the experiment. In one dog this plan was carried out completely as described. In two other dogs curves were obtained at less frequent intervals, and in one dog the experiment was terminated before edema appeared. In two of the dogs casein hydrolysate was also given by gavage, so that parallel studies were made with the amino acid given intravenously and by gavage.

### *Chemical Methods*

Minimal amounts of dry potassium oxalate were used as anticoagulant and the hematocrit values were estimated by the capillary hematocrit tube method (11). Plasma was analyzed for amino acid N by the gasometric ninhydrin method of Van Slyke, Dillon, MacFadyen, and Hamilton (10). Howe's technique (12) was used to separate albumin and globulin. Total protein and plasma non-protein nitrogen were determined by the micro-Kjeldahl technique as previously described (13). Blood urea was determined by the hypobromite method of Van Slyke and Kugel (14).

<sup>3</sup> Kindly furnished by Mead Johnson and Company.

The same method was used to estimate urea plus ammonia in the urine. The urea clearance calculated from these figures represents therefore the urea + ammonia clearance. Maximal urea clearances were calculated throughout, since Shannon (15) has shown that no augmentation limit occurs in the dog. Urinary ammonia was determined by aeration in the presence of potassium carbonate into  $N/70$   $HCl$ , a modification of the Folin method.

### *Results (Intravenous Casein Hydrolysate)*

*Plasma Amino Acid Retention*—In all of the dogs as hypoproteinemia developed the curves showed progressive retention of plasma amino acids following an injection of casein hydrolysate. The complete data are presented in Table I. Following a control intravenous injection of saline while the dog was fed a normal diet, the fasting level of 4.29 mg. per 100 cc. fell slightly, then rose—without, however, reaching the preinjection level within 3 hours. These changes are not accounted for by the fall in hematocrit (Table I). With the dog still on a normal diet an intravenous injection of casein hydrolysate showed a sharp rise in the plasma level; so that 5 minutes after the injection the level was recorded at 6.2 mg. per 100 cc., then dropped rapidly, and had reached the preinjection level in about 20 minutes.<sup>4</sup> A further drop occurred paralleling the control injection with saline.

The dog was then fed the low protein diet and 1 week later the injection was repeated. During this week the plasma albumin fell from 3.17 to 2.61 gm. per 100 cc. Following the injection the plasma amino acids rose higher and were cleared from the blood stream less rapidly, so that the preinjection level was not reached until 1 hour later. That retention could be shown so early in the course of hypoproteinemia was a complete surprise. Thereafter at intervals further loading curves showed progressive increase in the retention with one exception. During the 3rd week (Table I) on the low protein diet Dog 958 refused to eat and the curve at this time fails to show retention. The changes in the liver are probably reversible at this stage of hypoproteinemia (8) but it is not clear how failure to accept carbohydrate and fat could produce a favorable change. Whatever the cause, this is the only instance in all the studies on three dogs in which successive periods on the low protein diet failed to show progressive sluggishness of amino acid removal from the blood stream. After 20 weeks on the low protein diet the albumin had fallen to 1.37 gm. per 100 cc. and slight edema of the ankles appeared. Plasma amino acid retention at this time was maximal.

<sup>4</sup> Estimates of the time required for the serum amino acid concentration to return to the preinjection level are approximate. They were obtained by interpolation along the charted curve of the analytical observations.

TABLE I

*Plasma Amino Acid N, Blood Urea N, and Hematocrit Values before and after Injections of Casein Hydrolysate in Normal and Hypoproteinemic Dog 958*

Plasma amino acid N and blood urea N are expressed in mg. per 100 cc., the hematocrit values in per cent.

Wks. on diet	Plasma amino acid N					Blood urea N					Hematocrit										
	Before		After injection, min.			Before		After injection, min.			Before		After injection, min.								
			5	15	30			90	180	5			15	30	90	180					
Normal diet																					
4	2.98*	4.20	4.00	3.39	2.99	3.31	3.64	10.60	12.04	9.70	9.27	9.04	10.06	44.5	41.3	44.5	41.3	40.7	11.4	38.9	37.9
4	3.17	4.14	6.20	4.51	3.34	2.59	3.06	9.48	10.46	11.21	10.46	9.55	8.50	40.5	38.4	40.5	38.4	40.7	11.4	38.9	37.9
Low protein diet																					
1	2.61	4.44	7.36	5.68	4.91	4.21	3.64	5.98	6.19	6.86	5.30	4.32	5.23	43.1	41.4	45.6	41.4	45.6	43.8	44.4	41.0
2	2.34	5.00	8.42	6.19	5.25	4.65	4.12	3.95	3.46	3.26	3.78	3.54	3.15	46.7	40.5	41.7	41.4	45.6	41.4	40.9	37.6
3	2.16	4.89	7.08	5.28	4.48	3.44	3.60	3.11	2.65	2.96	3.74	2.79	3.07	44.1	44.5	46.3	45.0	45.4	43.8	41.6	41.1
4	2.11	4.79	8.99	7.37	5.60	4.46	3.63	5.72	5.70	5.72	5.26	4.01	4.62	45.6	44.0	42.4	42.3	45.4	43.8	40.3	39.3
5	2.12	4.57	9.12	7.11	5.09	3.65	3.43	4.33	4.06	5.21	3.86	5.04	4.78	49.3	42.0	45.0	45.4	45.4	43.8	44.7	
8	1.84	3.97	8.27	6.65	4.67	3.78	3.07	3.69	5.08	3.48	3.99	3.83	3.12	47.3	40.2	42.5	44.1	45.4	43.8	41.2	40.5
13	1.67	4.02	9.07	6.82	5.06	3.42	3.71	2.01	2.94	2.51	3.05	2.33	1.95	43.8	40.3	40.6	40.8	40.8	38.3	39.0	
20	1.37	4.39	10.94	8.12	6.34	4.51	3.90	4.07	4.07	4.95	4.85	4.02	4.15	31.1	31.4	32.6	33.1	33.1	31.3	30.9	

\* Control injection of 5 per cent glucose in saline.

\* Control injection of 5 per cent glucose in saline.

Many years ago Van Slyke and Meyer (16) demonstrated that amino acids given intravenously are removed very rapidly from the blood stream. Our experiments confirm these findings. In a normal dog of 10 kilos the preinjection level of plasma amino acid N was 4.1 mg. per 100 cc. After an intravenous injection of 120 mg. of amino acid N, calculations on the basis of an assumed blood volume of 960 cc. (17) indicate that the total circulating plasma amino acid N should be about 144 mg. if no amino acid had been removed from the plasma. The determined plasma amino acid N 5 minutes after the injection was only 6.2 mg. per 100 cc., which indicates that about 108 mg. or 90 per cent of the amino acids had already been removed from the plasma. 15 minutes after injection more than 98 per cent had disappeared. The rapid disappearance of the amino acid is undoubtedly due to absorption by the tissues.

During the course of experimental hypoproteinemia gradual loss of weight and rapid reduction in plasma volume occur (17). Since in our experiments the total dosage of amino acid given (20 cc. of 10 per cent casein hydrolysate) did not vary throughout the course of hypoproteinemia, it might be questioned whether the retention of plasma amino acids was actual or merely represented the result of injecting more amino acid N per kilo of body weight into an animal with a smaller blood volume. As shown in the calculation above, the retention of plasma amino acid following intravenous injection of casein hydrolysate in the normal dog was of the order of 10 per cent at 5 minutes and less than 2 per cent at 15 minutes. After 2 weeks on the low protein diet no loss of weight had occurred; so the amount of amino acid N given per kilo of body weight was not increased. However, the reduction in plasma volume was appreciable, about 100 cc. as interpolated from a previously determined composite curve (17). On the basis of this estimate of the plasma volume, the retention of plasma amino acid after 2 weeks of low protein feeding was calculated to be 17 per cent at 5 minutes, 7 per cent at 15 minutes, and 2 per cent at 30 minutes. After 5 weeks on the diet with a weight loss of 0.9 kilo, indicating a slight increase in the amount of amino acid N given per kilo of body weight and a further reduction in plasma volume of about 108 cc. (interpolation), the plasma amino acid retention was 17.7 per cent at 5 minutes, 9.5 per cent at 15 minutes, and 2 per cent at 30 minutes. These calculations serve as confirmatory evidence that the retention is actual, and not spurious or merely relative.

*Relation between Fasting Plasma Amino Acid and Serum Albumin*—It was rather a surprise to find that the value of plasma amino acid during fasting was maintained at a normal level in spite of the severe degree of muscle wasting which occurred as hypoproteinemia progressed and furthermore that there was no correlation between plasma amino acid values

during fasting and the fall in the plasma albumin concentration. In Fig. 1 the plasma amino acid N levels are compared with a composite albumin curve during the development of hypoproteinemia in three dogs. In one dog (No. 308) the plasma amino acid N level remained normal during the first 6 weeks on the low protein diet and dropped below the normal when massive generalized anasarca had developed at 20 weeks with a plasma albumin level of 1.22 gm. per 100 cc. Since no intervening studies were made, it is not known exactly when the fall occurred. In another dog (No. 771) the plasma amino acid level was still normal after 9 weeks on the low protein diet with a plasma albumin of 1.53 gm. per 100 cc. At this time the experiment was terminated.<sup>5</sup> In the third dog (No. 958) the plasma amino acid N was still within normal limits after 20 weeks on the low protein diet when the plasma albumin had fallen to 1.37 gm. per 100 cc.

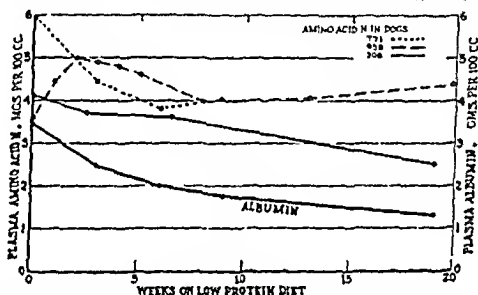


FIG. 1. Comparison of plasma amino acid N levels with a composite curve of plasma albumin values during the course of hypoproteinemia in dogs.

*Metabolism Studies*—As hypoproteinemia developed, the blood urea fell, rapidly at first and then more slowly. The normal value of 10 mg. per 100 cc. dropped to 6 mg. after 1 week on the low protein diet, and after 2 weeks it had reached 4 mg. Thereafter the urea level fluctuated around this value. However, in the individual experiments during the observation period of 3 hours following the intravenous injection, although the amino acids were metabolized, there was no significant change in the blood urea level. This is probably accounted for by the prompt excretion of urea.

Metabolism studies for 3 hours following the injection of casein hydrolysate were carried out as hypoproteinemia developed. The data from the experiments on one dog (No. 958) are assembled in Table II. The results

<sup>5</sup> The high normal value of 6 mg. per 100 cc. in this dog was obtained during high ment feeding. Previously determined values on the standard normal diet in this dog had likewise been about 4 mg. per 100 cc.

were confirmed by similar studies on one other dog. While the dog was on a normal diet, a control injection of 5 per cent glucose in saline was given. The plasma amino acid N levels fell following the injection and had not returned to the preinjection value in 3 hours (Table I). The amounts of urea, ammonia, and amino acid excreted were remarkably constant throughout the period of observation (Table II). The urea clearance fluctuated somewhat during the experiment, while the amino acid clearance was minimal. Then with the dog still on the normal diet an injection of casein hydrolysate was given. The plasma amino acid N level rose and then dropped below the preinjection level, the fall paralleling the results after the control injection of saline. Following the injection of amino acid, excretion of ammonia and urea was increased. The bulk of the increase in ammonia excretion occurred during the first half hour after the injection, while the increase in urea excretion appeared later, for the most part during the last period.

After the dog was placed on the low protein diet, metabolism studies were made at intervals throughout the course of hypoproteinemia. We were particularly interested in learning whether, with progressive protein starvation and presumably with progressive impairment of liver function, the ability to metabolize amino acids would be adversely affected and whether ammonia and urea formation would still be possible. The conservation of body nitrogen was apparent very early in the course (Table II). However, no change was noted in the manner in which amino acids given intravenously were metabolized during the entire course of deproteinization. Increases in ammonia and urea excretion occurred whenever an injection was given, although the amounts excreted decreased progressively, paralleling the decline in serum albumin. It is apparent that the hypoproteinemic dog does not lose the ability to form ammonia and urea from available amino acid.

The value of the ratio, ammonia N + urea N to total N, in urine was 0.81 during the control experiment when an injection of 5 per cent glucose in saline was given in the fasting normal dog. The ratio remained the same (0.83) following an injection of casein hydrolysate in the fasting normal dog. During the course of hypoproteinemia the ratio, although slightly lower, ranging from 0.70 to 0.75, remained almost constant throughout the course (Table II). The constancy of the ratio suggests that the material was metabolized in the same fashion throughout.

The urinary studies furnished an indirect approach to an estimation of the rate of deamination. The quantity of total N excreted before the injection during the control period was considered to represent basal catabolism. The excretion of total nitrogen during the first 3 hours after the injection was assumed to result from basal catabolism plus metabolism of



TABLE II

*Urinary N Excretion Immediately Following Injections of Casein Hydrolysate in Normal and Hypoproteinemic Dog 958*

Wks on diet    Metabolism period No.    Total time    Urine pH    Urinary excretion    Ammonia N + urea N\*    Urine volume    Clearances

Wks on diet	Metabolism period No.	Total time	Urine pH	Urinary excretion				Ammonia N + urea N* Total N	Urine volume	Clearances	
				Urea N	Ammonia N	Amino acid N	Total N			Urea	Amino acid
Normal diet											
		min.		mg. per 30 min.	mg. per 30 min.	mg. per 30 min.	mg. per 30 min.		cc per min.	cc per min. per sq m.	cc per min. per sq m.
4	1†	0-42		60.5	0.4	0.5	75.6	0.81	0.24	37	0.9
	2	42-95		79.9	0.5	0.4	96.7		1.08	56	0.8
	3	95-183		72.0	1.7	0.9	91.2		2.91	48	1.6
4	Control	0-44	7.22	56.4	1.7	0.4	74.3	0.83	0.25	38	0.6
	1†	44-82	5.28	69.2	6.9	1.7	107.7		0.25	41	
	2	82-136	6.72	95.8	3.2	0.6	119.0		0.69	60	1.4
	3	136-227	7.55	101.5	4.4	0.6	121.5		3.80	71	1.3
Low protein diet											
1	Control	0-47	7.7	25.1	1.3	0.4	44.7	0.74	1.45	27	0.7
	1†	47-76	6.8	13.4	2.8	0.3	38.2		0.59	13	
	2	76-139	7.2	41.0	1.6	0.4	62.1		1.65	54	0.6
	3	139-227	7.3	49.1	2.8	0.5	62.1	0.72	2.42	57	0.9
2	Control	0-41	8.03	18.8	1.2	0.3	28.8		1.05	30	0.4
	1†	41-80	7.07	11.8	2.4	2.0	31.0		0.60	21	
	2	80-134	6.94	26.7	2.3	0.6	41.1	0.70	1.52	46	0.7
	3	134-224	7.58	26.6	1.6	0.4	34.2		2.13	50	0.6
3	Control	0-43	7.35	15.6	1.7	0.2	24.7	0.70	0.98	32	0.3
	1†	43-97	6.25	9.8	3.0	0.6	24.2		0.85	20	
	2	97-146	6.43	18.5	2.6	0.4	32.0		0.96	36	0.6
	3	146-254	7.31	19.0	3.0	0.2	27.5	0.73	1.91	42	0.4
4	Control	0-39	8.23	19.4	0.8	0.2	28.8		0.72	22	0.3
	1†	39-75	7.31	17.3	2.7	1.7	42.2	0.73	0.44	20	
	2	75-132	7.18	31.2	2.7	0.5	43.6		2.65	43	0.6
	3	132-235	7.29	23.7	2.6	0.3	32.9	0.75	1.85	35	0.4
5	Control	0-40	7.88	16.4	1.2	0.3	24.2		0.65	25	0.4
	1†	40-93	6.85	9.7	1.5	2.3	28.4	0.75	0.16	15	
	2	93-147	6.77	24.4	1.8	0.4	37.3		0.56	33	0.6
	3	147-244	7.10	31.2	3.2	0.3	37.1	0.74	2.81	42	0.6
8	Control	0-32	7.08	15.2	1.3	0.3	21.1		0.38	28	0.4
	1†	32-62	6.09	11.6	3.4	2.1	26.4	0.74	0.37	18	
	2	62-120	6.40	16.1	2.4	0.5	25.5		0.66	27	0.7
	3	120-217	7.18	15.2	2.5	0.3	21.6	0.70	2.06	29	0.6
13	Control	0-39	7.77	11.2	0.6	0.1	16.5		0.91	38	0.2
	1†	39-89	7.07	8.7	1.8	1.8	26.4	0.70	0.72	23	
	2	89-139	7.84	17.0	1.7	0.4	26.8		0.56	43	0.7
	3	139-231	7.67	19.2	3.3	0.2	25.6		2.89	61	0.3

TABLE II—*Concluded*

\* Ratio, total amounts after intravenous injection.

† Control intravenous injection of 5 per cent glucose in saline.

‡ Intravenous.

the injected amino acid. If from the total determined N excreted after the injection the estimated amount of total N due to catabolism (from extrapolation of the control period) is deducted, the result can be considered to represent the metabolism of the injected material alone. Calculations made on this basis for total N and its fractions, urea N and ammonia N, afford an estimate of the nitrogen excretion due to the metabolism of the injected casein hydrolysate throughout the entire course of hypoproteinemia. As early as 1 week after the dog was fed the low protein diet a marked reduction could be observed in the excretion of urea N, ammonia N, and total N due to the injected load. A further fall took place during the 2nd week of deproteinization, and thereafter the values persisted at the low level. These experiments indicate that the rate of deamination was greatly reduced in the hypoproteinemic dog.

*Urea and Amino Acid Clearance*—The clearances calculated from the data are also recorded in Table II. The fluctuations in urea clearance following the control injection of 5 per cent glucose in saline were similar to the fluctuations observed after injections of casein hydrolysate. Apparently the quantity of amino acid injected was not sufficient to produce a consistent elevation of the urea clearance. An increase in urea clearance caused by protein feeding in the normal dog was demonstrated many years ago by Jolliffe and Smith (18) and by Van Slyke *et al.* (19). Recently Pitts (20) has shown that the injection of certain amino acids has a similar effect. Under the conditions of our experiments no rise was observed which could be related to the injection of casein hydrolysate.

At no time during the course of the experiments was there sufficient excretion of amino acids to yield an amino acid clearance above a very low value. All amino acid clearances were calculated to be under 2 cc. per minute per sq.m. (Table II). The plasma amino acid N in these experiments did not rise above 11 mg. per 100 cc., and was maintained at this level for brief periods only.<sup>6</sup>

#### *Results (Casein Hydrolysate by Gavage)*

In order to investigate the reaction of the hypoproteinemic dog with liver injury to amino acid received in a more physiologic fashion, experiments in which amino acids were introduced by tube into the stomach were carried out on two of the dogs. The dogs were given casein

<sup>6</sup> A more complete study of the amino acid clearance will be presented elsewhere.

hydrolysate, about 110 mg. of amino acid N per kilo, and observed through the successive 5 hours. Blood samples were obtained before and at 15 minutes, 30 minutes, 1, 2, 3, and 5 hours after the gavage. From one of the dogs urine was collected by catheter at suitable intervals for investigation of the metabolism of the material.

As in the experiments when casein hydrolysate was injected intravenously, increasing plasma retention occurred as hypoproteinemia progressed. In the normal dog there was no rise in plasma amino acid N until after the 1st hour, a moderate rise during the 2nd and 3rd hours, and then a fall, so that at the 5th hour the preinjection value was regained. After 3 weeks on the low protein diet (in these experiments observations earlier in the course of hypoproteinemia were not made) retention was present at 5 hours. After 6 and 9 weeks on the low protein diet the plasma amino acid N was still rising at 5 hours, even though the value was twice the normal value. Curiously, following the gavage a gradual drop in plasma amino acid N occurred during the first 2 hours. The blood urea values during each experiment fluctuated within a narrow range without noticeable trend in direction. No difference was noted in the manner in which the casein hydrolysate was metabolized whether the material was given intravenously or by gavage. The metabolism studies following gavage were carried out on Dog 771. Throughout the deproteinization, in this case of 9 weeks duration, the metabolism of amino acids was evidenced by increased ammonia and urea excretion in the urine, in like manner to the experiments in which the amino acid was administered intravenously.

#### *Results (Single Amino Acids)*

Before the experiments recorded here were begun, several of the available single amino acids were tested for comparison with casein hydrolysate. One dog (No. 308) received parallel injections of glycine and casein hydrolysate throughout the course of the deproteinization until edema appeared. The curves were almost identical and both sets showed progressive retention. Urinary excretion studies were not carried out. Single observations with *dl*-alanine likewise paralleled the results with glycine and casein hydrolysate. On the basis of these preliminary studies casein hydrolysate was selected as a satisfactory source of mixed amino acid.

*Pathology*—The liver sections from Dog 771, sacrificed after 9 weeks on the diet, resembled those described by Elman and Heifetz (9). The hepatic cells were swollen and showed extreme vacuolation. The sinusoids were narrow. However, in contrast to the findings of Elman and Heifetz, stains with Sudan III showed a moderate amount of fat within the cells. Kidney sections on this animal revealed little or no morphological changes except that the stain with Sudan III also showed a moderate amount of

fat present within the tubules. The sections from Dogs 308 and 958 which developed edema after 19 and 20 weeks, respectively, on the low protein diet revealed practically no abnormal morphological findings in either the liver or the kidney, although the retention of plasma amino acid was like that in the two other dogs.

The pathological studies were kindly made by Dr. Beryl H. Paige and Dr. Dorothy H. Andersen of Babies Hospital.

#### DISCUSSION

The experiments reported here demonstrate for the hypoproteinemic dog an increased retention of plasma amino acid during the 90 minute period following an intravenous load of casein hydrolysate. That impairment of liver function results in such an experimental animal was substantiated by Elman and Heifetz (9). They detected impairment of liver function as early as the 16th day on the low protein diet and morphological changes in 3 weeks. The morphological changes consisted of extensive vacuolation of the liver cells, which was considered to be due to a loss of liver protein rather than an accumulation of either fat or glycogen. The severity of the morphological changes paralleled the fall in albumin as did liver function measured by the excretion of iso-iodoikonic acid. Previous studies from this laboratory indicate that not all dogs rendered hypoproteinemic show morphological changes (7, 8). In two of the dogs reported here, Dogs 308 and 958, practically no morphological changes were demonstrated, although the plasma amino acid retention equaled that in the other animal, Dog 771, with morphological injury. Biological variation from unknown accessory causes may account for the differences in the degree of morphological change in the livers of different hypoproteinemic dogs.

At the present time it is commonly accepted that practically all deamination occurs in the liver unless acidosis creates an unusual demand for ammonia formation by the kidney (21-23). If renal deamination occurs, the chances on the basis of organ size indicate that it will prove to be of relatively small quantitative importance (5).

The level of the plasma amino acid in the blood is conditioned by several factors, (1) the intake, (2) the immediate diffusion and storage in the liver and other tissues, (3) the rate of deamination in the liver, and (4) the release of stored quantities in the tissues until equilibrium is again attained. The manner in which amino acids are bound temporarily by the tissues is not known. Van Slyke and Meyer (16, 22) demonstrated that the combination must be in the nature of a loose physicochemical union, since they were able to free amino acids from tissues by the simple procedure of tri-

turation with water. They likewise demonstrated that the tissues, muscle, liver, and kidney, of normal dogs were able to retain large quantities of amino acids. Still less is known concerning the rôle of muscle in the hypoproteinemic dog. It is generally agreed that normal muscle stores relatively little amino acid and that only enough amino acid is converted into tissue protein in the adult animal to replace wear and tear. It is logical to assume that the need of hypoproteinemic muscle and liver is much greater and that more amino acid would be diverted to the synthesis of new protein. Analyses kindly made by Dr. D. D. Van Slyke of the tissues, muscle, liver, and kidney, of Dog 958, sacrificed after 20 weeks on the low protein diet, showed that the amino acid content was only slightly lower than normal. The plasma amino acid N level during fasting was similarly normal throughout most of the course.

The following argument is offered in an attempt to explain the retention of plasma amino acids found in hypoproteinemic dogs after an intravenous injection. Amino acids enter the plasma and diffuse into and are absorbed by muscles, liver, and other tissues. In the liver most of the amino acid is deaminized and excreted as urea; a portion is probably used for the synthesis of liver protein. In the muscle, however, part of the amino acid is converted into protein and the rest is returned to the plasma to make its way eventually to the liver to be deaminized there. In the normal dog these forces are balanced in such a fashion that if 12 mg. of amino acid N per kilo as casein hydrolysate are injected intravenously the plasma level is elevated for about 20 minutes only. However, metabolism of the injected amino acid continues long after the plasma level has fallen to normal, as is shown by the continuous excretion of urea above the level of catabolism.

An increased plasma value might be expected if either or both of these two avenues were blocked; *i.e.*, if muscle tissues were unable to absorb amino acids so quickly or if the liver deaminized more slowly. If the muscle tissues were more avid for amino acid because more amino acid was needed for the synthesis of new tissue protein, as might logically be assumed in hypoproteinemia, the plasma amino acid level would fall even more quickly than in the normal state. However, in the hypoproteinemic dog, observations indicate that after only 1 week on the deficient diet, the preinjection level is not reached until 1 hour after the intravenous administration. The delay in attainment of equilibrium increases as deproteinization progresses. Since the intake of amino acid was the same, and since it might be expected that muscle and liver in the hypoproteinemic dog utilize more amino acid for the synthesis of new tissue protein, the inference is strong that the retention of plasma amino acid was caused by a slower rate of deamination. With the realization that many undeter-

mined factors complicate the picture the experiments nevertheless offer an indirect approach to an estimation of the rate of deamination. The amounts of urea N and ammonia N formed as the result of metabolism of the injected material, exclusive of catabolism of body protein, demonstrate that as early as 1 week after deproteinization was begun only one-third of the expected amount was excreted by the hypoproteinemic dog. A further decrease occurred during the 2nd week and thereafter the excretion of ammonia N and urea N fluctuated around a low level. This reduction occurred in spite of the increased plasma amino acid N level which presumably offered available amino acid to the liver. Since the ratio of ammonia N + urea N to total N was constant throughout the course of hypoproteinemia, it may be inferred that the casein hydrolysate was used as a metabolic protein. Support is thus offered for the suggestion that the plasma amino acid retention may be due largely to reduction in the rate of deamination in the hypoproteinemic dog.

Retention of plasma amino acid was demonstrated equally well whether the casein hydrolysate was given intravenously or introduced into the stomach by tube. If the load is given by gavage a long period of observation is necessary, at least 5 hours after the ingestion of amino acid, to observe plasma retention. Our experiments confirm Kirk's (5) suspicion that the glycine tests he carried out on patients with liver disease failed to show plasma amino acid retention because the observations were limited to 2 hours after ingestion.

The constancy of the plasma amino acid level during fasting in spite of the severe depletion of serum albumin and the marked muscle wasting was not expected. In nephrosis both serum albumin and plasma amino acid are low for long periods of time, as shown by Farr and MacFadyen (24). Hence it was assumed that the fall in serum albumin in the hypoproteinemic dog might be accompanied by a parallel fall in plasma amino acid.

#### SUMMARY

In the hypoproteinemic dog when casein hydrolysate is given intravenously or by gavage, the plasma is not cleared of amino acid so rapidly as in the normal dog. The plasma amino acid retention is obvious after only 1 week on the low protein diet and increases progressively during the course of deproteinization. The retention is probably the result of reduction in the rate of deamination in an impaired liver.

In hypoproteinemic dogs no correlation was found between the plasma amino acid N level and the plasma albumin value. The plasma amino acid N level is maintained at the normal value for long periods in the face of severe hypoalbuminemia, sometimes until edema appears.

The metabolism of casein hydrolysate given intravenously or by gavage remains essentially unchanged throughout the course of progressive hypoproteinemia. An increase in ammonia and urea excretion occurs. Most of the ammonia is excreted immediately, while there is a short lag in the excretion of the greater part of the urea.

Under the conditions of the experiments the intravenous injection of casein hydrolysate failed to stimulate urea clearance in the hypoproteinemic dog as well as in the normal dog. With plasma amino acid N levels under 11 mg. per 100 cc. only small amounts of amino acid were excreted; so that the amino acid clearance was minimal throughout the course of deproteinization.

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# A GREEN PIGMENT-PRODUCING COMPOUND IN URINE OF PYRIDOXINE-DEFICIENT RATS

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While determining the bisulfite-binding substance of urine from pyridoxine-deficient rats and their paired pyridoxine-fed controls, E. J. Wickson, a student assistant, noticed that when slightly alkaline the urine of the pyridoxine-deficient rat had a green color, making the iodometric titration in the determination of bisulfite-binding substances impossible. The green pigment was found only in the urine of rats deficient in pyridoxine. It was not excreted as such, but as a precursor which became green upon contact with the iron of the metabolism cages. This color could also be readily developed by the addition of ferric ammonium sulfate and other iron salts to the urine. The pigment was green only while the pH was near the neutral point. The green color disappeared upon the addition of either acid or alkali and reappeared upon neutralization.

Administration of pyridoxine to the deficient rat caused the disappearance from its urine in 6 to 10 hours of most of the precursor of the green pigment, indicating a close relationship between it and pyridoxine deficiency.

The green pigment was concentrated by chromatographic procedures with filter paper as the packing in the column. It acted as an indicator, being light yellow in strongly acid solution, green when near neutrality, and yellow in strongly alkaline solution. Qualitative tests of the compound indicated the presence of carbon, hydrogen, nitrogen, and iron.

*Care of Animals*—The animals were fed the pyridoxine-deficient diet previously employed (1) with the following changes: Factor 2 concentrates were discontinued and in their place were used 200  $\gamma$  daily of Ca pantothenate<sup>1</sup> (Merck) and a rice bran preparation to supply unknown filtrate factors. It was prepared by extracting a rice bran concentrate (Vitab rice bran concentrate,<sup>2</sup> Nopco) with acetone. The acetone extract was concentrated and the sugar was fermented out of the concentrate with yeast. The alcohol was removed by distillation *in vacuo*, and the concentrate made strongly acid with H<sub>2</sub>SO<sub>4</sub> (Congo red) and extracted continuously with ether for 1 week. The extracted concentrate was diluted

<sup>1</sup> Kindly furnished by Merck and Company, Inc., Rahway, New Jersey.

<sup>2</sup> Kindly furnished by The Vitab Corporation, Emeryville, California.



with about 4 volumes of water and extracted twice with fullers' earth and twice with charcoal (Nuchar C45). The amount of adsorbing agent used each time equaled about half the weight of the concentrate. An acid reaction was maintained throughout. The extract was then filtered and concentrated. Such extracts carried little pyridoxine or pantothenic acid and 0.25 cc. was fed daily to each rat.

The rats were fed the pyridoxine-deficient diet (1) *ad libitum* and were kept in round iron metabolism cages 8.5 inches deep and 9 inches in diameter. The base of the cage was a screen, two meshes to the inch and large enough to allow the feces to fall through. The cage was set on an iron funnel to collect the urine. A finer mesh screen was placed on the funnel to prevent the feces and large food particles from going down the neck of the funnel. No attempt was made to keep the urine free of all contamination, since it did not seem to interfere with the production of the green pigment.

*Concentration of Pigment*—The urine from the pyridoxine-deficient rats was adjusted approximately to neutrality and ferric ammonium sulfate was added. This turned the urine a dark green. The pH was kept at about 8 in order to remove excess ferric ion as the hydroxide. All suspended material was removed by centrifugation or filtration. The green urine was saturated with NaCl and poured onto a paper-packed chromatographic column, on which the pigment was adsorbed. The column was then washed with saturated NaCl to remove all the urine, and the pigment then was eluted with distilled water. The green eluate was concentrated under reduced pressure until the salt crystallized out. Care must be taken that concentration is carried out in neutral or slightly acid solutions. An equal volume of ethanol and 8 volumes of acetone were then added. The precipitate, consisting mostly of sodium chloride, was centrifuged out and the solution again fractionated on the column. The chromatogram was then developed with ethanol, which slowly carried the pigment down. The pigment was again eluted with distilled water and carefully taken to dryness. A drop of a concentrate of this pigment when put on the slide evaporated slowly and took on characteristic patterns, as shown in Fig. 1.

*Color and pH*—To the solution of the purified green pigment, both acid and alkali were gradually added and the pH and color changes were noted. At pH 3.5 or less, the pigment was a very light yellow. At pH 3.5 to 5.5 there was a transition from light yellow to green. The green color persisted between pH 5.5 and 11.3, when the color went through a transition from green to yellow between pH 11.3 and 12.3. Above 12.3 the color of the pigment was yellow. The pH measurements were made with the glass electrode.

**Solubility**—The pigment is readily soluble in water, fairly soluble in alcohol, and insoluble in pure acetone. It is, however, somewhat soluble in high concentrations of acetone. It is insoluble in ether.

**Stability**—The pigment is quite stable to heat in acid or neutral solution, but less stable in basic solution. It is stable in acetone solution when



FIG. 1. Green pigment patterns; I  $\times 54$ ; II  $\times 45$ ; III  $\times 225$

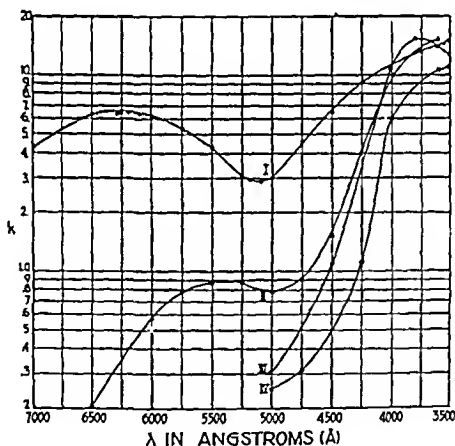


FIG. 2. Absorption spectra of the green pigment. Curve I, neutral solution; maximum at 6250 Å.,  $k = 6.52$ ; Curve II, reduced solution; maximum of shelf about 5300 Å.,  $k = 0.90$ ; Curve III, basic solution; 0.1 N  $\text{OH}^-$ ; maximum at 3800 Å.,  $k = 15.8$ ; Curve IV, acidic solution; 0.1 N  $\text{H}^+$ ; no maximum above 3500 Å.

purified but very unstable in acetone in the presence of an unidentified constituent of normal urine. Many normal constituents of urine, including urea, uric acid, creatine, creatinine, cholic acid, desoxycholic acid, and acetic acid have been tested, but none of these decolorizes the green pigment in acetone solution. Oxidizing agents readily decolorize the pigment.

*Behavior toward Reducing Agents*—Sodium sulfite will not decolorize the green pigment, but sodium hydrosulfite readily decolorizes it. When adjusted to a pH just turning phenolphthalein, the solution is a brownish red. On the addition of acid it becomes a deep yellow in the neighborhood of neutrality and a light yellow in acid solution (Congo red). The green color can be restored with weak solutions of hydrogen peroxide or by shaking in air.

*Absorption Spectrum*—The absorption spectrum of the compound in neutral, acid, and alkaline solution is shown in Fig. 2. These curves were obtained by Mr. S. Aronoff and Dr. G. Mackinney of the Division of Fruit Products. The curve is also shown for the hydrosulfite-treated compound with the pH adjusted to give a brownish red color to the solution.

#### DISCUSSION

Since pyridoxine seems essential for hemoglobin formation (2), it seemed possible that the compound studied might be a porphyrin or related product. The Gmelin and Ehrlich diazo tests for bilins were negative. No fluorescence was observed in the visible region. These properties when considered with the absorption spectrum of the compound cannot be reconciled with a porphyrin structure.

#### SUMMARY

1. Pyridoxine-deficient rats excrete an unidentified compound which can be converted to a green pigment by ferric ammonium sulfate.
2. The pigment acts as an indicator to acids and bases, turning a light yellow in strongly acid solution and yellow in strongly basic solution.
3. Administration of pyridoxine to the deficient rats stops the excretion of the precursor of the green pigment in a matter of hours.
4. Some of the properties of the green pigment are discussed.

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# THE ELECTROPHORETIC PROPERTIES OF SERUM PROTEINS

## II. OBSERVATIONS ON FRACTIONS OF CRYSTALLINE HORSE SERUM ALBUMIN

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Numerous investigations have been carried out during the past decades dealing with the preparation and properties of crystalline horse serum albumin. While several modifications of the classical methods and conditions for the crystallization of this protein and its components have been introduced in recent years (1-6), the electrophoretic properties of any one fraction have not yet been investigated over a wide pH range with the refined optical methods for recording boundary shape. Although the electrophoretic mobility of horse serum albumin has been determined at pH regions other than that of the physiological range of about 7.6, a detailed resolution of the boundaries into their component parts was not possible with the experimental methods that were employed (2, 7-9).

The present paper describes an electrophoretic analysis of fractions of crystalline serum albumin, prepared according to Kekwick (2), carried out with the Tiselius electrophoresis apparatus in conjunction with the Thoevert-Philpot-Svensson optical system. Boundary anomalies seen in slightly acidified solutions of these proteins prompted measurements on a number of other preparations obtained by the methods of crystallization summarized in a recent publication from this laboratory (5). Comparative measurements on native protein and on the same material after apparent reversal of denaturation by concentrated urea solutions (9) are also recorded in this paper.

### *Material*

*Serum Albumin, Fractions A and B*—These crystalline fractions were obtained by fractional precipitation of fresh, pooled horse sera with sodium sulfate at 30° by the method of Kekwick (2). Fraction A contained, after three crystallizations, 1.95 per cent carbohydrate, whereas Fraction B was essentially carbohydrate-free. The behavior of these materials with respect to diffusion and viscosity has been described previously (5).

For comparative electrophoretic measurements various other crystalline fractions were prepared (5). These fractions are related to each other in

that their isolation involved fractional precipitation with ammonium sulfate under controlled conditions of pH and salt and protein concentration. They were essentially free of carbohydrate but differed from each other in solubility in ammonium sulfate and in some details of the purification process.

*Serum Albumin SA<sub>I</sub>*—This material was prepared according to Hewitt's method (1) with the modifications described previously (5). The protein was recrystallized seven times; in the last crystallization precipitation occurred from a 2 per cent protein solution of pH 5.5, between 2.3 and 2.5 M ammonium sulfate.

*Serum Albumin SA<sub>a</sub> and SA<sub>III</sub>*—McMeekin (3) described a method according to which carbohydrate-free preparations of crystalline horse serum albumin could be separated into two fractions, one of which was precipitable from concentrated solutions made to pH 4.0 with sulfuric acid (SA<sub>a</sub>). It was shown subsequently (5) that only that fraction of crystalline horse serum albumin least soluble in ammonium sulfate was capable of yielding such a water-insoluble protein sulfate and that this protein, although monodisperse, differed from the other crystalline albumin fractions with respect to diffusion and viscosity.

The serum albumin SA<sub>a</sub> employed in the present work was the same as that used in the previous studies (5). It was derived from a fraction which in the third recrystallization was precipitated from a 10 per cent protein solution by 1.6 M ammonium sulfate at pH 5.5. The more soluble fraction, precipitating in the third recrystallization at 1.9 M ammonium sulfate at pH 5.5, did not yield a water-insoluble protein sulfate. This fraction (SA<sub>III</sub>) was crystallized once more.

*Apparently Reversibly<sup>1</sup> Denatured Serum Albumin*—This protein was prepared from Fraction A as described before (10). Denaturation was effected by dissolving the protein in 8 M urea solutions; the apparently reversibly denatured fraction was reprecipitated twice from a 3 per cent protein solution of pH 5.2 by adding 23 gm. of sodium sulfate to 100 ml. of protein solution.

Monovalent acetate, veronal, and mixed acetate-veronal buffers were employed. Solutions of 0.1 ionic strength were prepared as described before (11), with the difference that acetic acid instead of hydrochloric acid was used for adjusting the acetate buffers to the desired ionic strength. Solutions of 0.02 ionic strength were prepared as follows: Between pH 7.7 and 5.96, 0.820 gm. of anhydrous sodium acetate and 2.061 gm. of sodium veronal were weighed out for each liter of solution. The salts were dissolved in a volume of distilled water slightly less than 1000 ml. To the

<sup>1</sup> See (10) foot-note 2.

solution was added an amount of 5.0 N hydrochloric acid necessary to produce the desired pH and the total volume was then increased to 1000 ml. Between pH 5.38 and 3.50, 1.611 gm. of anhydrous sodium acetate were similarly dissolved, an appropriate volume of 5.0 N acetic acid was added, and the total volume made to 1000 ml.

### Results

The electrophoretic patterns and mobilities of Fractions A and B were determined over a pH range from 7.7 to 3.5.

The following description of the electrophoretic patterns relates to Fraction A in solutions of 0.1 ionic strength. At pH 7.6, the protein migrated with a single, symmetrically shaped boundary, as illustrated in Fig. 1, *a*. A single boundary was also observed at pH 6.8; however, the refractive index gradient curves were slightly skewed with both the ascending and descending boundary. At pH 6.0, there was seen a slight break in the advancing edge of the descending boundary, while the ascending boundary was symmetrical, although slightly skewed as in the preceding experiment. A reversal of the order of boundary inhomogeneity occurred when the pH was lowered to 5.38. Here, the descending boundary showed a distinct split which was reflected in the appearance of the ascending boundary merely as a slight break in its receding edge (see Fig. 1, *b*). In solutions slightly acid with respect to the isoelectric point the electrophoretic patterns appeared to be utterly anomalous. The pattern obtained at pH 4.4 is recorded in Fig. 2, *a*. The ascending boundary showed two distinct peaks, while the descending boundary failed to exhibit a corresponding resolution. In more acid solutions, *e.g.* at pH 3.6, boundary homogeneity was regained; the boundaries were single, although slightly skewed, as shown in Fig. 1, *c*. The boundary patterns seen with Fraction B in solutions of 0.1 ionic strength were similar to those just described for Fraction A. While slight differences were observed in the extent to which minor boundaries developed (*e.g.* at pH 5.38), they were not sufficient to warrant a differentiation between Fractions A and B on the basis of their electrophoretic patterns.

Electrophoretic measurements in solutions of 0.02 ionic strength also showed the boundary patterns to be alike for Fractions A and B, over the whole pH range investigated. At pH 7.7 the protein migrated with a single, symmetrically shaped boundary, just as it did at pH 7.6 and 0.1 ionic strength. At pH 6.0 the ascending boundary was split, while the descending boundary revealed only a faint indication of a second peak, as can be seen in Fig. 1, *d* for Fraction A, and in Fig. 1, *e* for Fraction B. As in the measurements in solutions of 0.1 ionic strength, reversal of the order of boundary inhomogeneities occurred when the pH was lowered to 5.38.

Under this condition the descending boundary showed a break in the receding edge, while the ascending boundary was symmetrical. At pH

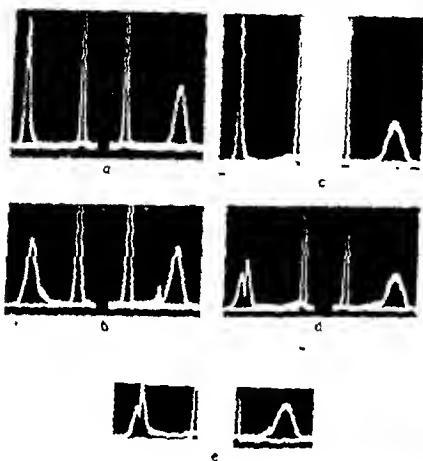


FIG. 1

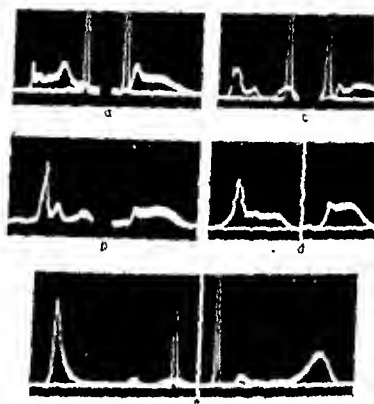


FIG. 2

FIG. 1. Electrophoretic patterns of crystalline horse serum albumin. The left-hand side of each picture represents the curves of the refractive index gradient of the ascending boundary, the right-hand side those of the descending boundary. The sharp peaks near the center indicate the positions of the starting boundaries. (a) Fraction A at pH 7.6 and 0.1 ionic strength; 149 minutes migration at 4.37 volts per cm. (b) Fraction A at pH 5.38 and 0.1 ionic strength; 213 minutes migration at 5.16 volts per cm. (c) Fraction A at pH 3.6 and 0.1 ionic strength; 200 minutes migration at 3.50 volts per cm. (d) Fraction A at pH 5.98 and 0.02 ionic strength; 150 minutes migration at 5.07 volts per cm. (e) Fraction B at pH 5.96 and 0.02 ionic strength; 150 minutes migration at 4.97 volts per cm. Due to accidental displacement of the photographic plate, the base-line does not fuse into the pattern in this particular diagram.

FIG. 2. Electrophoretic patterns of fractions of crystalline horse serum albumin. The left-hand side of each picture represents the curves of the refractive index gradient of the ascending boundary, the right-hand side those of the descending boundary. The sharp peaks near the center indicate the positions of the starting boundaries. (a) Fraction A at pH 4.4 and 0.1 ionic strength; 332 minutes migration at 4.38 volts per cm. (b) Fraction A at pH 4.4 and 0.02 ionic strength (starting boundary not shown); 95 minutes migration at 7.58 volts per cm. (c) Fraction SA<sub>1</sub> at pH 4.4 and 0.02 ionic strength; 98 minutes migration at 7.09 volts per cm. (d) Fraction SA<sub>2</sub> at pH 4.4 and 0.1 ionic strength (starting boundaries not shown); 286 minutes migration at 4.66 volts per cm. (e) Fraction A after exposure to 70°, for 120 minutes at pH 4.1; measurements at pH 4.2 and 0.1 ionic strength; 288 minutes migration at 5.81 volts per cm.

4.8, the reported isoelectric point of the protein (2), the major peaks of the boundaries were stationary, whereas a small fraction of the descending boundary could be seen to migrate very slowly toward the anode. In the

pH range of 4.4 to 4.0 the ascending and descending boundaries did not show the remotest similarity. At pH 4.4 (Fig. 2, *b*) the descending boundary appeared as a flat plateau, falling off sharply at the receding edge and

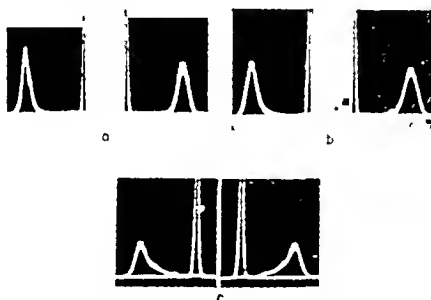


FIG. 3. Electrophoretic patterns of serum albumin Fraction A after apparently reversible denaturation by 8 M urea. The left-hand side of each picture represents the curves of the refractive index gradient of the ascending boundary, the right-hand side those of the descending boundary. The sharp peaks near the center indicate the positions of the starting boundaries. Measurements were in solutions of 0.1 ionic strength. (a) pH 7.6; 240 minutes migration at 5.22 volts per cm. (b) pH 6.0; 246 minutes migration at 3.38 volts per cm. (c) pH 5.38; 122 minutes migration at 9.45 volts per cm.

TABLE I

*Electrophoretic Mobilities of Horse Serum Albumin Preparations in Solutions of 0.1 Ionic Strength*

The mobilities reported in this paper have the dimensions, sq. cm. per second per volt.

pH	Mobility $\times 10^5$			
	Fraction A	Fraction B	Fractions A and B	Fraction A "reversibly" denatured by 8 M urea
7.60	-5.97	-6.18	-6.15	-6.57
6.80	-5.17			
6.00	-4.24	-4.40		-4.86
5.38	-3.14	-3.31		-3.36
	-2.07*			
4.40	Anomalous			
4.20	"	Anomalous		
3.60	5.07	5.36	5.34	5.35

\* Small secondary boundary.

gradually at the advancing edge, whereas the ascending boundary revealed two distinct peaks. At pH 4.0, the ascending boundary also revealed a double peak, while the descending boundary remained unresolved. At



pH 3.5, the proteins migrated with a single, slightly skewed boundary, of the type shown in Fig. 1, *c*.

In an attempt to decide whether the boundary anomalies observed for Fractions A and B of the serum albumin around pH 4.4 were a specific property of these particular fractions or a characteristic generally associated with the horse serum albumins measurements were carried out at the same pH and ionic strength on Fractions SA<sub>I</sub>, SA<sub>α</sub>, and SA<sub>III</sub>. Fig. 2, *c* shows the electrophoretic pattern for Fraction SA<sub>I</sub> at pH 4.4 and 0.02 ionic strength and a similar pattern was obtained for Fraction SA<sub>III</sub>. In Fig. 2, *d*, an analogous pattern is shown for Fraction SA<sub>α</sub> at pH 4.4 and 0.1 ionic strength. It will be noted that the diagrams shown in Fig. 2, *a* and *d*, are all of the same type although of slightly different details. Such complex electrophoretic patterns could also be seen in the albumin fraction of whole horse serum when subjected to electrophoresis at pH 4.4 and 0.1 ionic strength. These electrophoretic anomalies disappear upon exposure of the protein solutions to higher temperatures. In one experiment, a 2 per cent solution of Fraction A was adjusted to pH 4.1 and heated for 120 minutes to 70°. After cooling, an acetate buffer of pH 4.2 and 0.1 ionic strength was added and the solution equilibrated against this buffer by dialysis. As may be seen from Fig. 2, *c*, the principal components are present in both the ascending and descending boundary, whereas the untreated protein revealed no such similarity at this pH and ionic strength.

A more complete account of the electrophoretic properties of the heat-treated serum albumin fractions will be published elsewhere.

The electrophoretic patterns of Fraction A after apparently reversible denaturation by 8 M urea are shown in Fig. 3, *a*, *b*, and *c*, as obtained in solutions of 0.1 ionic strength at pH 7.6, 6.0, and 5.38, respectively. Comparison with the native protein (Fig. 1, *a* and *b*) reveals a close similarity at pH 7.6 but a slightly different behavior at pH 5.38.

In Table I the data relative to pH and electrophoretic mobility of the various fractions, as measured in solutions of 0.1 ionic strength, are given. Mobilities were calculated from the maximum ordinate of the descending boundaries, as these have been shown to be more nearly correct (12).

#### DISCUSSION

Profound changes in electrophoretic pattern of horse serum albumin occur with variations of pH. While in pH regions remote from the isoelectric point, such as 7.6 and 3.6, the protein migrates with a single boundary, the boundaries become complex as the pH of the solution approaches that of the isoelectric point. This complex behavior is manifest not only in boundary splitting but also in a lack of similarity of ascending and descending boundary. In solutions of 0.02 ionic strength

at pH 6.0 only the ascending boundary is resolved into a double peak, while at pH 5.38 only the descending boundary is split. At pH 5.2, the ascending boundary assumes a better resolution and appears to preserve it throughout the acid pH range. A similar trend was observed with the measurements in solutions of 0.1 ionic strength. However, small variations in the boundary pattern do occur with variations in ionic strength as may be seen, for instance, from the descriptions given for the experiments at pH 6.0 in solutions of 0.1 and 0.02 ionic strength.

It may be noted also that the relative amount of total area comprised by the faster moving component varies appreciably with pH.

The electrophoretic anomalies become most prominent in pH regions around 4.4. In these measurements, the ascending boundary, where the protein ions migrate into the buffer, consists of at least two peaks; practically no analogous resolution could be observed with the descending boundary where the protein ions migrate into a solution containing ions of their own kind. The similarity in these patterns and that observed for the albumin fraction of whole serum at pH 4.4 suggests that this complex behavior is not due to secondary changes in the protein occurring as a result of purification but rather a property inherent in *native* horse serum albumin.

The presence of two electrophoretically different albumin components in normal and pathological sera and in urinary proteins at pH 4.0 has been observed by Luetzsch (8), using the simple Toepler schlieren method for visual observation of the boundary position. However, in view of the present observations in this pH range, it is doubtful whether any significance can be attached to ascending boundaries which have no counterpart at the descending side (see for instance Fig. 2, *b*). It is also apparent that fractional crystallization at pH 4.0, under the conditions described by McMeekin (3), does not alter significantly the electrophoretic pattern of the protein. Thus, Fraction SA<sub>2</sub> revealed about the same complex boundary appearance (Fig. 2, *d*) as did the protein from which it was derived (Fig. 2, *c*).

The electrophoretic mobilities of Fractions A and B are essentially the same throughout the entire pH range. This is borne out not only by a comparison of the mobilities of the isolated fractions but also by measurements on solutions containing mixtures of both proteins. In these experiments, solutions containing equal amounts of Fractions A and B were subjected to electrophoresis in pH regions where the isolated fractions migrated with single boundaries. No boundary separation could be observed and the mobilities of the mixtures were practically identical with those of the component parts (Table I). Although Kewick (2), using the light absorption method for recording boundary position, and working

at 20°, observed small differences in the mobilities of these two fractions, it is questionable whether they are significant in the face of the limited resolving power of his method. The same considerations may apply also to the data of Moyer and Moyer (9), obtained from microscopic electrophoresis measurements on coated quartz or collodion particles.

The protein obtained after apparent reversal of denaturation by 8 M urea has a slightly higher mobility than the native on the alkaline side of the isoelectric point but practically the same mobility on its acid side. The electrophoretic patterns reveal also a greater amount of boundary spread than those seen with the native protein. These differences, together with those already described (10, 13), emphasize the essentially irreversible nature of the denaturation process under the conditions that were employed.

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#### SUMMARY

The present paper describes an electrophoretic analysis of fractions of crystalline horse serum albumin carried out with the Tiselius apparatus and the Thoevert-Philpot-Svensson optical system for recording boundary shape. The electrophoretic pattern and mobility of Fractions A and B prepared according to Kekwick's method have been investigated over a range of pH 7.7 to 3.5, in solutions of 0.1 and 0.02 ionic strength. Both fractions revealed a complex boundary pattern in a pH range between 4 and 6 which was distinguished by a lack of symmetry between the ascending and descending boundaries. This was particularly pronounced near pH 4.4 and was observed also with other crystalline albumin fractions as well as with the albumin of whole, untreated horse serum. It is concluded that these anomalies reflect a complex nature of horse serum albumin in general and were not caused by secondary changes produced during the purification process. The electrophoretic mobilities of Fractions A and B were found to be the same over the entire pH range. This was also confirmed by measurements on mixtures of both fractions.

The protein obtained by apparent reversal of denaturation by 8 M urea solutions was found to differ from the native protein in electrophoretic mobility on the alkaline side of the isoelectric point, and in a greater amount of boundary spread. The differences observed here together with those

already described indicate that under the conditions employed the denaturation of serum albumin is irreversible.

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# A SPECTROPHOTOMETRIC METHOD FOR THE ANALYSIS OF CHLOROPLAST PIGMENTS

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An investigation of the pigmentation of *Chlorella pyrenoidosa* has required a method enabling rapid changes in the pigment concentrations to be followed. Adaptations of the classical method of Willstätter and Stoll, involving partition of the pigments between solvents, are time-consuming and, as pointed out by many authors, are frequently inaccurate. The separation of the groups of chloroplast pigments by chromatographic adsorption currently in use by Egle (1), Seybold and Egle (6), and others is not strictly quantitative, as is indicated by Mackinney (2) and by experience in this laboratory. Furthermore, the chromatographic method requires too much time to enable one to follow rapid changes in pigmentation.

A new method has been developed which permits each of the four groups of chloroplast pigments to be determined with speed and accuracy. It is a modification and extension of a spectrophotoelectric method described by Zscheile (9). Working with mixtures of chlorophylls *a* and *b* in solution, Zscheile measured the light absorption at two wave-lengths. At one of these wave-lengths both chlorophylls have the same absorption coefficient and at the other there is a great difference in the two coefficients. From these measurements it was possible to calculate the amounts of chlorophylls *a* and *b* in the solution with an error of less than 1 per cent. Employing the same principle, Miller (5) analyzed solutions of  $\beta$ -carotene and leaf xanthophyll with equal success.

*Principle of Method*—In the present method, a sample of material is extracted with hot methyl alcohol, and the pigments are transferred to ether. The carotenes present in an aliquot are separated by adsorption on a sugar column and estimated directly from measurements made with a calibrated Pulfrich photometer with appropriate filters (No. S-47 or S-50).

Chlorophyll *a*, chlorophyll *b*, and total carotenoids are determined from measurements of the extinction coefficients of the extract made in three different regions of the spectrum. To estimate the quantities of chlorophylls *a* and *b*, two filters are employed (Nos. S-61 and S-66) which transmit wave-lengths which are not absorbed by the carotenoids but which are absorbed in different degree by chlorophylls *a* and *b*. From calibration curves for the extinction of each of these spectral bands by pure chlorophylls *a* and *b* (Fig. 1), a diagram has been constructed (Fig. 2) from which the

concentrations of chlorophylls *a* and *b* present in a mixture may be read directly when the extinction of both bands is known.

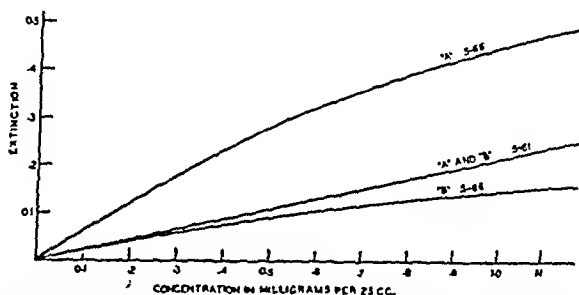


FIG. 1. Calibration curves of Pulfrich photometer for chlorophylls *a* and *b*; 5 mm. cell; filters as indicated on the curves. Extinction =  $\log I_0/I_x$ .

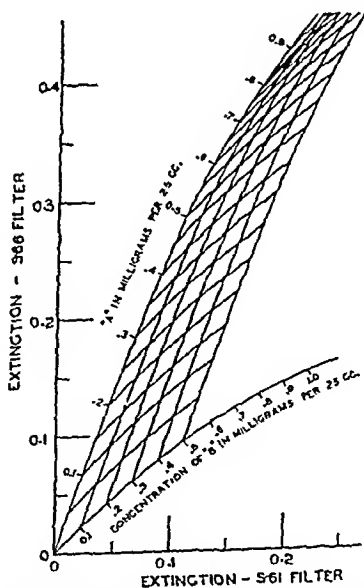


FIG. 2. Concentration-extinction grid for chlorophylls *a* and *b* in ether; 5 mm. absorption layer. The grid is plotted with values obtained from the curves shown in Fig. 1. Extinction =  $\log I_0/I_x$ .

To obtain the concentration of xanthophylls, the extinction is measured with a spectral band absorbed maximally by the carotenoids (Filter S-47 or S-50). Calibration curves are made showing the extinction of this band

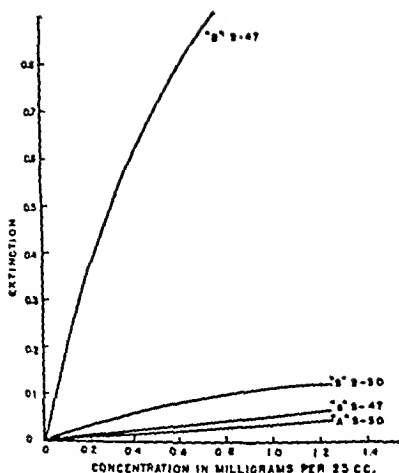


FIG. 3

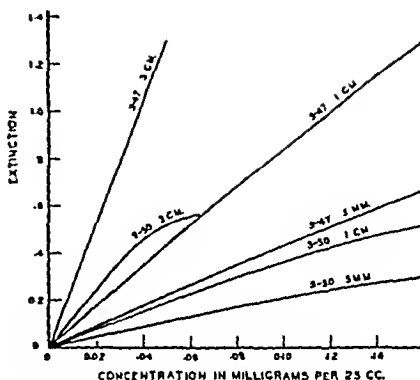


FIG. 4

FIG. 3. Calibration curves of Pulfrich photometer for chlorophylls *a* and *b*; 5 mm. cell; filters as indicated on the curves. Extinction =  $\log I_0/I_x$ .

FIG. 4. Calibration curves of Pulfrich photometer for carotenes in petroleum ether. Absorption layer thicknesses and filters as indicated. Extinction =  $\log I_0/I_x$ .

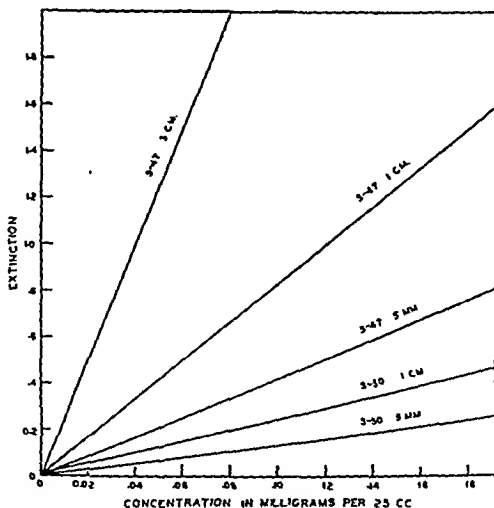


FIG. 5. Calibration curves of Pulfrich photometer for xanthophylls in ether. Absorption layer thicknesses and filters as indicated. Extinction =  $\log I_0/I_x$ .

by pure samples of each of the groups of pigments (Figs. 3, 4, and 5). By the aid of these curves and the estimations of the concentrations of carotene



and chlorophylls *a* and *b*, the extinction of this band due to each of these components may be estimated. Subtracting these values from the total extinction by the extract gives the extinction due to the xanthophylls. From this value the concentration of xanthophyll is obtained.

In the method it is assumed that practically all light absorption, within the range of the filters used, is by the chloroplast pigments. This seems justified, for never in the partition or chromatographic methods have colored substances not identifiable as chloroplast pigments been observed. Since no colored material has ever been discarded in transfer from one solvent to another or in the wash solution, all the pigments extracted from the cells have been accounted for as chloroplast pigments. Water-soluble pigments are eliminated by washing from the ether solution before absorption measurements are made. Since the development of this method, Mackinney (4) has shown that the light absorption by crude leaf extracts in the visible spectrum above 5300 Å. can be completely accounted for by chlorophyll alone.<sup>1</sup>

*Detailed Description of Method. Extraction of Pigment*—A very rapid method of killing and extraction with hot methyl alcohol has been used. Although various workers have objected to the use of heat in the extraction of leaf pigments, results obtained in the extraction of *Chlorella* cells are not significantly different whether hot solvent extraction or extraction in the

<sup>1</sup> All calibration curves of the Pulfrich photometer were made with pigment preparations previously checked for purity on the Hardy automatically recording photoelectric spectrophotometer available for use at the Massachusetts Institute of Technology, Color Laboratory. The pigment standards were obtained from various sources:  $\alpha$ -carotene, a crystalline preparation of General Biochemicals, Inc., Cleveland;  $\beta$ -carotene and leaf xanthophyll, crystalline preparations of The American Chlorophyll Company, Inc., Alexandria, Virginia; chlorophylls *a* and *b*, prepared in this laboratory according to the method of Mackinney (3). These were checked spectroscopically against preparations kindly sent by Professor Mackinney. Our two chlorophyll *b* preparations appeared to be identical. My chlorophyll *a* gave approximately one-third lower specific absorption values than Professor Mackinney's, as though my preparation had contained a colorless impurity. The characteristics of the filters of the Pulfrich photometer are as follows:

Filter No	Transmission maximum	Effective center of gravity	Effective breadth
	Å.	Å.	Å.
S-66	6800	6650	320
S-61	6200	6190	200
S-50	4900	4960	230
S-47	4580	4650	260

The effective breadth is the range of wave-lengths within which transmission is greater than 50 per cent of the maximum.

cold with grinding is employed. This is true only if the heating of the material with the solvent is not prolonged and if the extract is cooled quickly after filtering. Although hot methyl alcohol treatment has proved satisfactory in extracting a limited number of plants in this laboratory, one must not minimize the importance of the statement by Spoehr, Smith, Strain, and Milner (7) that when one considers the diversity of leaf structures no one method can be invariably applicable, and that a method should be critically examined for possible flaws when used on a particular plant.

When *Chlorella* cells are extracted, duplicate samples of the culture containing a convenient amount (usually 30 to 60 mg. of dry weight) are centrifuged until the cells are packed so that the supernatant medium can be poured off. The cells are resuspended in 10 cc. of 98 per cent methyl alcohol and brought nearly to the boiling point by immersing the centrifuge tubes in boiling water. The hot suspension of cells is poured over a sintered glass filter (Jena No. 4G) and the cells are sucked dry. A major portion of the pigment is removed in this first step. The filtrate is cooled immediately by the immersion of the suction flasks in an ice bath during the filtration.

The residue of dry cells is scraped into a mortar and ground thoroughly with 0.5 to 0.8 mg. of finely powdered quartz sand. 98 per cent methyl alcohol is added in the final stages of grinding, and the mixture is washed quantitatively back into the centrifuge bottle. After the mixture is rewarmed by brief immersion of the bottle in boiling water, it is poured back on the filter. This second extraction practically always removes all traces of pigment from the cells. As a precautionary measure, warm methyl alcohol to make a total volume of extractant of 50 cc. is used to wash the grayish cell residue on the filter, and this is followed by 50 cc. of ethyl ether.

*Preparation of Pigments for Spectrophotometric Analysis*—The total pigment extract is washed quantitatively into a 250 cc. separatory funnel with an additional 50 cc. of ether. Cautious addition of distilled water down the side of the funnel will drive the pigments up to the ether layer practically quantitatively. The lower aqueous methyl alcohol layer is washed with successive 10 cc. portions of ether until the wash layer is absolutely colorless. The combined ether extracts (usually 50 to 75 cc.) are in turn freed of methyl alcohol by five rinsings with equal volumes of distilled water. The ether solution is transferred to a small Erlenmeyer flask and is reduced to about 20 cc. volume by aspiration at 20–25°. If anhydrous sodium sulfate for drying the ether solution is added prior to aspiration, it will prevent bumping.

The water-free ether solution is filtered quantitatively into a 50 cc. volumetric flask and is brought up to volume with fresh ether used to rinse the Erlenmeyer flask and sodium sulfate.

The final results of the analysis (in mg. per sample) are as follows:

	Sample 1	Sample 2	Per cent difference
Chlorophyll <i>a</i> .....	0.782	0.808	3.1
" <i>b</i> .....	0.232	0.218	6.1
Xanthophyll, Filter S-50.....	0.109	0.106	3.0
"    "    S-47.....	0.117	0.123	5.0
Carotenes.....	0.0175	0.0178	1.7

The dry weight of the *Chlorella* cells in these samples was 39.7 mg.

*Results of Analyses of Known Mixtures*—To test the dependability of the spectrophotometric method, mixtures of known amounts of the chlorophylls and carotenoids were prepared and analyzed. A typical experiment gave the results shown in Table I. Such experiments show that the spectrophotometric method is applicable to the analysis of mixtures of chloroplast

TABLE I  
*Pigments from Known Mixtures by Spectrophotometric Method*

Sample No.		Chlorophyll <i>a</i>	Chlorophyll <i>b</i>	Xanthophyll
		mg. per 25 cc.	mg. per 25 cc.	mg. per 25 cc.
1	Present	0.563	0.210	0.075
	Found	0.550	0.220	0.072
	Difference, %	-2.6	+0.5	-4.0
2	Present	0.885	0.405	0.076
	Found	0.874	0.400	0.086
	Difference, %	-1.2	-1.2	+12.0

pigments. The accuracy of the method is far greater than that reported for analyses of known mixtures by the chromatographic method.

*Comparison with Results of Other Methods*—For comparison with the results published by other workers and obtained by the more time-consuming and arduous techniques, various plant materials have been analyzed by the spectrophotometric method. The comparative results are given in Table II.

In considering these results, one must remember that all the variations are not due to differences in analytical method. The pigment content of plant materials will vary according to the environmental conditions under which the plants were grown. Differences in handling the materials prior to pigment extraction will also give variations. It is improbable, for example, that analytical errors alone would account for the large range of values found by Seybold and Egle in their analyses of six series of *Ulua lactuca* (see Table II).

TABLE II

Comparison of Results of Analyses of Leaf Pigments by Spectrophotometric, Chromatographic, and Partition Methods

The results are measured in mg. per 10 gm. of fresh weight.

Method		Pigment concentrations and ratios						
		a	b	x	c	$\frac{a}{b}$	$\frac{x}{c}$	$\frac{a+b}{x+c}$
<i>Fagus silvatica</i> *								
Partition, Willstätter and Stoll	Sun	26.76		1.86	3.13	1.63	3.45	
	Shade	37.40		3.83	2.92	1.92	6.02	
Chromatographic, Egle (1)	Sun	36.5	9.8	2.71	1.86	3.7	1.5	6.2
	Shade	42.4	14.2	4.08	0.58	3.0	7.1	7.4
Spectrophotometric	Sun	26.85	7.65	3.25	1.37	3.5	2.4	4.7
	Shade	36.2	12.7	3.175	1.70	2.8	1.9	6.26
<i>Lemna minor</i>								
Chromatographic, Egle (1)		7.2	2.2	1.06	0.38	3.2	2.8	4.0
Spectrophotometric	Sam- ple 1†	9.5	3.38	1.02	0.443	2.8	2.3	5.5
	Sam- ple 2‡	10.3	3.85	1.23	0.435	2.7	2.8	5.32
<i>Ulva lactuca</i>								
Chromatographic, Seybold and Egle (6)	§	9.3	4.1	2.18	0.44	2.3	5.0	3.1
Spectrophotometric	Sam- ple 1	18.7	11.07	3.57	1.04	1.7	3.4	5.15
	Sam- ple 2	20.2	11.90	3.81	1.16	1.7	3.3	4.03

a and b = chlorophylls a and b, x = xanthophyll, and c = carotene.

\* Green leaves; grown in sun or shade as indicated.

† Plants taken from aquarium in greenhouse.

‡ Plants grown in full nutrient medium in these laboratories by Mr. David Kaufman.

§ The results given here are the average of six published series. The extremes of the six vary from one another by over 100 per cent in the amount of chlorophyll found and nearly as much in the other pigments.

|| Material collected on tide flats at Nahant, Massachusetts, and extracted while fresh.

*Limitations of Method*—The Pulfrich filters have a broad spectral range of transmission, and no available red filter covers a band in which the chlorophyll b absorption is higher than that of chlorophyll a. With the two

filters employed the concentration-extinction curves for chlorophyll *b* are so flat (Fig. 1) that small errors in reading the extinction give large errors in determining the chlorophyll *b*.

Successive readings of the extinction of the same sample give values which vary as much as 2 per cent from the mean. The corresponding estimates of chlorophyll concentration are  $\pm 3$  per cent for chlorophyll *a* and  $\pm 6$  per cent for chlorophyll *b*. This illustrates the extreme sensitivity of the determined chlorophyll *b* value to slight errors in the extinction measurements.

Unfortunately, chlorophyll *b* absorbs light strongly in the region of maximum transmission of Filter S-47. Thus errors in determination of the chlorophyll *b* result in serious errors in the corrections applied to the reading obtained with Filter S-47 for the determination of the xanthophylls. Abnormally high chlorophyll *b* values will lead to apparent xanthophyll values that are abnormally low, and conversely.

To minimize such errors in the analysis of chlorophyll *b*, which influence greatly the values obtained for the xanthophylls present, extinction measurements are also taken with Filter S-50. In this spectral range, the xanthophylls still exhibit a strong absorption of light (about one-third that in the range of Filter S-47), but the absorption by chlorophyll *b* is cut to about one-fifth that in the range of Filter S-47. This means that with Filter S-50, as contrasted to Filter S-47, the effect of an error in the chlorophyll *b* value on the xanthophyll value is cut nearly in half.

Use of Filter S-50 provides another valuable check. When pheophytin is present in the mixture of pigments, the absorption values with Filters S-66 and S-61 are reduced in proportion to the amount of pheophytin formation. As Tiegs (8) has pointed out, however, the absorption with Filter S-50 is increased by the presence of pheophytin. Therefore, appreciable pheophytin formation gives apparent xanthophyll values with readings with Filter S-50 that are higher than are determined with readings with Filter S-47. In such cases, gray pheophytin bands appear in the sucrose columns used to separate the carotenes from the other pigments. Unless the xanthophyll values, as determined by the use of Filters S-47 and S-50, check within 10 per cent, the analysis should be discarded as untrustworthy.

Certain precautions must be observed in drawing conclusions from an indirect method of analysis. Mackinney (2) points out that, when spectroscopic data indicate significant changes in the pigment content, supplementary evidence must be obtained to avoid ascribing such changes to erroneous causes. In the same paper Mackinney describes some of the spectroscopic changes that occur as the chlorophylls undergo certain decomposition reactions in the process of extraction. Shifts in absorption

maxima along the wave-length scale or changes in magnitude of extinction, such as are described, would alter the results of a spectrophotometric analysis on chlorophyll solutions containing small amounts of decomposition products significantly.

Owing to its great refinement, the method of Zscheile becomes susceptible to large errors through comparatively minor changes in the absorption properties of the solutions. With his monochromator system, Zscheile was able to use spectral bands only 7 Å. in width for extinction measurements. A slight shift of an absorption maximum in or out of this narrow band would invalidate the results of the analysis, and Mackinney (2) has demonstrated that such shifts can occur on chlorophyll decomposition. This objection has been met by using broader spectral bands for the extinction measurements. This procedure reduces the accuracy with which the components of a mixture can be distinguished, but at the same time it reduces the sensitivity of the method to slight changes in the absorption spectra of the components. The spectral bands of the Pulfrich photometer are of such breadth that slight decomposition of the chlorophyll makes no appreciable difference in the extinction values obtained.

In making quantitative measurements of chlorophylls, one cannot overlook the fact that no two workers agree completely on the spectroscopic properties of pure chlorophylls *a* and *b* in solution. This point has been reviewed by Mackinney (3) and still more recently by Zscheile and Comar (10). Therefore, it must be emphasized that the results given in this paper depend on the samples of chlorophylls used in the construction of the calibration curves for the optical instrument employed.

In spite of all limitations, the spectrophotometric method gives more accurate results on known mixtures of chlorophylls and carotenoids than either the partition or chromatographic method. The results of the analysis of duplicate samples of fresh plant material were also more consistent. The spectrophotometric method has been tested by use in an extensive series of experiments to be described elsewhere.

#### SUMMARY

A method for the quantitative estimation of the four major groups of leaf pigments, chlorophylls *a* and *b*, xanthophylls, and carotenes, is described, employing spectrophotometric measurement following extraction with hot methyl alcohol and isolation of carotene by adsorption on sugar.

The entire analysis may be completed in 6 hours. The results are concordant with those obtained by the partition and chromatographic technique but are more easily obtained and appear to be more precise when judged by the recovery of known mixtures of the purified pigments.

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# ON THE PROTEOLYTIC ENZYMES OF ANIMAL TISSUES

## IV. DIFFERENCES BETWEEN AEROBIC AND ANAEROBIC PROTEOLYSIS

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Previous experiments have shown that oxygen may affect the action of an intracellular proteolytic enzyme in two ways, either by oxidation of the activator or by acting on the enzyme protein itself (1). Enzymes that are activated by sulfhydryl compounds show a steady decrease in activity when the sulfhydryl group of the activator is oxidized by oxygen. If, in the course of this progressive action of oxygen, the oxygen is removed, no further decrease in proteolytic activity occurs but the original activity is not restored. On the other hand, certain enzymes, such as papain trypsinase, have been found to exist in two inactive forms which may be reversibly transformed into one another by oxidation-reduction processes.<sup>1</sup> In the presence of oxygen the reduced (HCN-activatable)  $\beta$  form is converted into the oxidized (HCN-inactivatable)  $\alpha$  form. If a solution of a  $\beta$ -enzyme is exposed to oxygen in the absence of sulfhydryl activators and samples are tested at intervals for proteolytic activity in the presence of HCN, a steady decrease in proteolytic activity will be observed due to a decrease in the amount of the  $\beta$  form of the enzyme. On removal of oxygen, no regeneration of the  $\beta$ -enzyme and consequently no regeneration of the proteolytic activity in the presence of HCN may be expected. In the present communication it is shown that, in addition to the two effects mentioned above, oxygen may influence proteolytic processes in still a third manner. In this case, the rate of proteolysis is spontaneously increased on removal of oxygen.

In the presence of an excess of cysteine, swine kidney carboxypeptidase hydrolyzes carbobenzoxyglycyl-L-phenylalanine<sup>2</sup> (2, 3). First order reaction constants are obtained for this hydrolysis in the presence as well as in the absence of oxygen. However, in nitrogen the substrate is hydrolyzed twice as rapidly as it is in air (Table I). At a higher oxygen tension, i.e. in a 1:1 oxygen-nitrogen atmosphere, the proteolytic rate is less than half of that found in air. The difference between the aerobic and anaerobic rates cannot be explained by the action of the oxygen upon the enzyme itself or upon the activator (cysteine).

<sup>1</sup> Irving, G. W., Jr., Fruton, J. S., and Bergmann, M., unpublished data. See also (1).

<sup>2</sup> In this paper carbobenzoxyglycyl-L-phenylalanine will be referred to as CGP.



When swine kidney carboxypeptidase was activated by cysteine in air and the activated enzyme solution was used for the hydrolysis of CGP in air, a proteolytic coefficient ( $C_{CGP}$ ) of 0.008 was obtained (Table II). However, when the activated enzyme was transferred from air to nitrogen immediately after the addition of the substrate, a higher constant rate of hydrolysis ( $C_{CGP} = 0.020$ ) was established at once.

In another experiment, the splitting of CGP by cysteine-activated swine kidney carboxypeptidase was allowed to go to completion in the presence of air ( $C_{CGP} = 0.009$ ). Two equal samples of the resulting solution were treated as follows: One sample was transferred to nitrogen, a second portion of substrate was added, and the rate of digestion was determined. The other sample was allowed to remain in air, a second portion of substrate

TABLE I

*Aerobic and Anaerobic Hydrolysis of Carbobenzoxyglycylphenylalanine (4) by Cysteine-Activated Swine Kidney Carboxypeptidase*

Enzyme preparation	Enzyme concentration, protein N per cc. test solution	Nitrogen		Air		$\frac{C(N_2)}{C(air)}$
		$K_{CGP}^*$	$C_{CGP}^*$	$K_{CGP}$	$C_{CGP}$	
	mg.					
A	0.179	0.0051	0.028	0.0025	0.014	2.0
B	0.205	0.0046	0.022	0.0010	0.0093	2.4
C†	0.202	0.0036	0.018	0.0018	0.0089	2.0
D	0.226	0.0060	0.027	0.0030	0.013	2.1
E	0.201	0.0046	0.023	0.0019	0.0095	2.4
F	0.198	0.0050	0.025	0.0023	0.012	2.1

\* These terms have been defined (5).

† Coefficients of 0.017 (anaerobic) and 0.0089 (aerobic) were found when the rate of hydrolysis was determined also by amino nitrogen measurements.

was added, and the rate of digestion was determined. The proteolytic coefficient in air was 0.008 and in nitrogen 0.018. This demonstrates that the amount of active carboxypeptidase remains unchanged throughout the course of the experiment and, consequently, that the retarding influence of air is not due to the oxidation either of cysteine or of the enzyme itself.

Direct titration of sulfhydryl groups showed no appreciable decrease in cysteine concentration in the aerobic experiments in the period during which rate constants were determined (Table III). It should be emphasized that the cysteine concentration employed represents a large excess.

Proportionality of reaction rate and enzyme concentration is known to exist for the aerobic action of swine kidney carboxypeptidase on CGP (2). It has now been found that this proportionality also holds under anaerobic conditions (Table IV).

In both the aerobic and anaerobic hydrolysis of CGP by carboxypeptidase, the same peptide bond is attacked. It has been found that after

TABLE II

*Hydrolysis of Carbobenzyglycylphenylalanine by Swine Kidney Carboxypeptidase; Effect of Transfer from Aerobic to Anaerobic Conditions*

A solution of swine kidney cathepsin was activated by cysteine for 2 hours at 40° in the presence of air. Carbobenzyglycylphenylalanine was added and part of the solution was immediately transferred to nitrogen; the remaining solution was allowed to remain in air. Proteolytic coefficients were determined for the aerobic and anaerobic hydrolysis. Enzyme concentration in test solution, 0.205 mg. of protein N per cc.

	Time of hydrolysis	$K_{CGP}$	$C_{CGP}$
	<i>min.</i>		
Nitrogen	34	0.0042	0.020
	70	0.0042	
	118	0.0043	
Air	38	0.0017	0.0083
	74	0.0016	
	122	0.0017	

TABLE III

*Determination of Sulfhydryl Groups during Aerobic and Anaerobic Hydrolysis of Carbobenzyglycylphenylalanine by Cysteine-Activated Swine Kidney Carboxypeptidase*

Enzyme concentration in test solution, 0.226 mg. of protein N per cc.

	Time of hydrolysis	$K_{CGP}$	$C_{CGP}$	Cysteine per cc. test solution*
	<i>min.</i>			<i>mM</i>
Nitrogen	0			0.019
	31	0.0063	0.027	
	60	0.0056		
	91	0.0061		0.020
	1200			0.019
Air	0			0.019
	40	0.0031	0.013	
	80	0.0031		
	116	0.0029		0.018
	1200			0.008

\* Determined by iodate titration (6). Cysteine added at start of experiment, 0.020 mM per cc.

complete hydrolysis identical amounts of the same split-products (carbobenzyglycine and phenylalanine) are formed in both cases. Moreover, it has been shown that the ratio between the rates of the anaerobic and

aerobic digestion of CGP is unchanged when the enzyme is partially inactivated by heat (Table V). It may be concluded, therefore, that the anaerobic and aerobic digestion of CGP is performed by the same carboxypeptidase and, further, that the lower reaction rate in air as compared with nitrogen can only be ascribed to the fact that a smaller amount of active enzyme exists under aerobic conditions.

TABLE IV

*Hydrolysis of Carbobenzoxyglycylphenylalanine by Cysteine-Activated Swine Kidney Carboxypeptidase*

	Enzyme concentration, protein N per cc. test solution	K <sub>CGP</sub>	C <sub>CGP</sub>
	mg.		
Nitrogen	0.113	0.0027	0.024
	0.170	0.0041	0.024
	0.226	0.0060	0.027
Air	0.113	0.0012	0.011
	0.226	0.0030	0.013
	0.452	0.0052	0.012

TABLE V

*Effect of Partial Inactivation by Heat on Anaerobic and Aerobic Activity of Cysteine-Swine Kidney Carboxypeptidase*

A solution of swine kidney cathepsin was heated at 50°. Samples were removed at the end of 5 and 12 minutes and proteolytic coefficients for the anaerobic and aerobic hydrolysis of CGP were determined. Enzyme concentration in test solutions, 0.226 mg. of protein N per cc. in aerobic experiments, 0.181 mg. in anaerobic experiments.

Period of heating at 50°	Nitrogen		Air		$\frac{C(N_2)}{C(air)}$
	K <sub>CGP</sub>	C <sub>CGP</sub>	K <sub>CGP</sub>	C <sub>CGP</sub>	
min.					
5	0.0017	0.0094	0.0011	0.0049	1.9
12	0.0011	0.0061	0.00076	0.0034	1.8

The activation of swine kidney carboxypeptidase by cysteine occurs so rapidly that no preliminary incubation period is required for the combination of the carboxypeptidase with cysteine. This is demonstrated by the fact that the anaerobic hydrolysis of CGP proceeds at a constant rate even when the incubation period is omitted (Table VI). However, in the aerobic digestion a constant reaction rate is obtained only if the enzyme is incubated with cysteine for some time before the substrate is added. If this

precaution is omitted, the aerobic digestion begins at almost as high a rate as the anaerobic digestion, but this rate diminishes as the hydrolysis proceeds. The fact that an incubation period is required to establish the constancy of the lower rate for aerobic hydrolysis indicates that in the course of the incubation cysteine reacts with a component of the swine kidney extract to form an inhibitor for the carboxypeptidase. This inhibitor is effective only under aerobic conditions.

The difference between the rates of aerobic and anaerobic proteolysis is not restricted to systems containing cysteine as the activator nor to swine kidney carboxypeptidase as the enzyme.

TABLE VI

*Influence of Activation Period of Swine Kidney Carboxypeptidase by Cysteine upon Aerobic and Anaerobic Hydrolysis of Carbobenzoxyglycylphenylalanine*

Enzyme concentration in test solution, 0.201 mg. of protein N per cc.

Period of incubation with cysteine, 40°	Nitrogen			Air		
	Time of hydrolysis	KCGP	CCGP	Time of hydrolysis	KCGP	CCGP
<i>min.</i>	<i>min.</i>			<i>min.</i>		
0	40	0.0049		40	0.0037	
	60	0.0049		60	0.0037	
	90	0.0051		96	0.0036	
	148	0.0050	0.025	182	0.0032	
120	40	0.0045		40	0.0021	
	60	0.0045		60	0.0020	
	90	0.0046		90	0.0018	
	150	0.0048	0.023	180	0.0018	0.010
180				40	0.0022	
				62	0.0021	
				90	0.0022	
				180	0.0019	0.010

When cysteine was replaced by glutathione as the activator for the swine kidney extract, CGP was hydrolyzed with a proteolytic coefficient of 0.020 in nitrogen and 0.010 in air. It is of interest that with HCN as the activator no difference in the rates ( $C_{CGP} = 0.017$ ) of the aerobic and anaerobic hydrolysis of CGP by the swine kidney extract was observed. The same enzyme preparation, when activated by cysteine, gave anaerobic and aerobic  $C_{CGP}$  values of 0.027 and 0.014 respectively.

A comparison of the rates of aerobic and anaerobic proteolysis by several intracellular enzymes is given in Table VII. The anaerobic proteolytic coefficients of cysteine-swine kidney trypsinase, cysteine-swine kidney aminopeptidase, and cysteine-beef spleen carboxypeptidase are signifi-

cantly higher than the corresponding aerobic coefficients. These enzymes are proteolytically active only in the presence of an activator. Swine kidney pepsinase, on the other hand, requires no activator and was found to show identical aerobic and anaerobic coefficients. This result is not surprising, since no sulfhydryl activator was present in this experiment and since, as shown above, a difference between the rates of anaerobic and aerobic proteolysis was observed only in the presence of cysteine or gluta-

TABLE VII

*Comparison of Aerobic and Anaerobic Proteolytic Coefficients of Several Intracellular Enzymes*

Enzyme	Enzyme concentration, protein N per cc. test solution	Substrate	Cysteine per cc. test solution	Nitrogen		Air		$\frac{C(N_2)}{C(air)}$
				K	C	K	C	
Swine kidney trypsinase	0.202	Benzoyl-L-arginineamide (7)	0.020	0.0051	0.025	0.0030	0.015	1.7
Swine kidney aminopeptidase	0.404	L-Leucylglycine	0.020	0.0012	0.0030	0.00036	0.0021	1.4
Beef spleen carboxypeptidase	0.344	Carbobenzoxylglycyl-L-phenylalanine	0.020	0.0019	0.0055	0.00090	0.0026	2.1
Swine kidney pepsinase*	0.253	Carbobenzoxyl-glutamyl-L-tyrosine (8)	None	0.00040	0.0016	0.00040	0.0016	1.0
Papain trypsinase†	0.0132	Benzoyl-L-arginineamide	0.020	0.0020	0.152	0.0019	0.144	1.1

\* Hydrolysis at 25°; all others at 40°.

† Thoroughly dialyzed against distilled water.

thione. It would have been desirable, therefore, to repeat the experiment with swine kidney pepsinase in the presence of cysteine or another sulfhydryl activator. However, such an experiment would not permit a simple interpretation, since, under these conditions, the substrate (carbobenzoxylglutamyltyrosine) would be attacked not only by the pepsinase but also by the carboxypeptidase present in the kidney extract (2). Consequently, a difference between aerobic and anaerobic proteolysis would be expected and it would be difficult to decide whether this difference could be attributed entirely to an aerobic inhibition of the carboxypeptidase or also, in

part, to a similar inhibition of the pepsinase in the presence of a sulfhydryl activator.<sup>3</sup>

In all of the experiments discussed thus far, 0.02 M citrate buffer (pH 5) was employed. When 0.02 M acetate buffer (pH 5) was used, the rate of hydrolysis of CGP by swine kidney carboxypeptidase was markedly lower and, furthermore, no difference in the rates of anaerobic and aerobic digestion was observed. When phthalate buffer (0.02 M, pH 5) was used, the rate of hydrolysis was again lower than that obtained with citrate; however, in this case, a more rapid hydrolysis was observed under anaerobic conditions (Table VIII).

The experiments reported in this paper indicate that, in the presence of oxygen and cysteine, swine kidney and beef spleen extracts form inhibitors which influence the activity of several proteolytic enzymes of these tissues. These inhibitors do not appear to be formed in the absence of oxygen. It is

TABLE VIII

*Effect of Various Buffers on Activity of Cysteine-Activated Swine Kidney Carboxypeptidase*

Buffer, 0.02 M, pH 5.0	Enzyme concentration, protein N per cc. test solution	Nitrogen		Air		$\frac{C(N_2)}{C(\text{air})}$
		K <sub>CGP</sub>	C <sub>CGP</sub>	K <sub>CGP</sub>	C <sub>CGP</sub>	
	mg.					
Citrate.....	0.198	0.0050	0.025	0.0023	0.012	2.1
Acetate.....	0.396	0.0015	0.0038	0.0014	0.0035	1.1
Phthalate.....	0.396	0.0021	0.0053	0.0014	0.0035	1.5

of interest that the same sulfhydryl compounds required in many cases for the formation of the active enzymes may also participate in the formation of inhibitors for these enzymes. The above experiments have demonstrated that these sulfhydryl compounds may act simultaneously in both capacities. It remains to be seen whether the mechanism by which changes in oxygen tension affect the action of sulfhydryl-activated proteolytic enzymes occurs in living tissues as well as *in vitro*.

The effect of oxygen tension upon proteolytic systems (such as papain + fibrin digest, or tissue extracts) has been studied by Maver and Voegtlin (9). It was concluded that proteolysis may be favored by the presence of sulfhydryl groups which on oxygenation may be converted into disulfide groups; the presence of the latter was assumed to favor protein synthesis. The experimental evidence for this conclusion has been

<sup>3</sup> With cysteine-papain-trypsinase, no marked difference between the aerobic and anaerobic rates was observed (Table VII).

disputed by Linderstrøm-Lang and Johansen (10). Since the experiments reported in this paper were performed with simple substrates, it would be premature to apply the results obtained to an interpretation of findings with substrates as complex as protein hydrolysates.

#### EXPERIMENTAL

The apparatus and procedure used in conducting enzymatic experiments under anaerobic conditions have been described previously (11). The aerobic experiments were performed either in this apparatus or in the usual manner in 2.5 cc. volumetric flasks. Except where otherwise indicated, hydrolyses were carried out at 40°; the test solution contained in each cc., 0.05 mm of substrate, 0.1 cc. of 0.2 M citrate buffer (pH 5.0), 0.020 mm of activator (cysteine or glutathione or HCN); the enzyme and activator were incubated for 2 hours at 40° before addition of the substrate. The extent of hydrolysis was determined by the titration procedure of Grassmann and Heyde (12).

The swine kidney and beef spleen enzyme preparations were obtained as described earlier (13). All preparations were dialyzed for 40 to 48 hours against 1 per cent NaCl at 4° before use. The papain preparation used was purified and thoroughly dialyzed against distilled water by the procedure given previously (1).

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# INVESTIGATIONS ON THE STABILITY OF AVIDIN

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An interesting characteristic of avidin (1), the constituent of egg white that is responsible for the production of egg white injury, is its stability under the varied conditions of the digestive tract. When avidin is fed to rats either in the form of raw egg white or as a purified concentrate, its continued activity is evidenced not only by the production of egg white injury but also by demonstration of its presence in the feces of animals suffering from egg white injury. In these feces it has been found that the biotin present is bound by avidin, since it becomes available for rats (2) and for yeast growth (3) only after steaming. This behavior is in contrast to the inactivation of avidin when it is administered parenterally. György and Rose (3) have shown that when avidin is injected into a rat suffering from egg white injury the disease is cured, presumably because bound biotin (present in all avidin so far prepared) is released and becomes available to the animal. Eakin, Snell, and Williams (4), in the presentation of their method of preparing avidin concentrates, drew attention to the fact that purified solutions of avidin may easily become inactivated. In this laboratory it was noted that avidin assays checked after several days frequently gave values that were considerably lower than those originally found. Woolley and Longworth (5), however, found that their preparations of avidin, which they call antibiotin, were quite constant in activity at ordinary temperatures. These last workers report also that antibiotin is resistant to inactivation by changes in pH, activity remaining constant from pH 1 to 11. This finding does not agree with the results of an earlier investigation by Parsons and Kelly (6), who found that the ability of dried egg white to produce egg white injury in rats was lost when it was treated with HCl at pH 2.4. In view of the low pH in the stomach, clarification of this point is of importance in any attempt to explain the physiological activity of avidin. The present study was undertaken to investigate further the factors involved in loss of avidin activity.

## EXPERIMENTAL

In all the experiments described the avidin concentrates were prepared and their activity was tested by the method of Eakin, Snell, and Williams (4).

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*Concentration and Temperature Tests*—Both concentration and temperature were found to be factors in the deterioration of avidin. An avidin preparation was dissolved in saline solution in four concentrations, 1, 0.1, 0.01, and 0.001 mg. per cc. The activity of this material (expressed in micrograms of biotin bound per mg.) as determined immediately was 7.5  $\gamma$ . Within 24 hours the activity of the most dilute solution kept in the refrigerator was only 1.9  $\gamma$  and kept at room temperature, 0.8. The activity of the 0.01 mg. solution after 5 days at refrigerator temperature was 4.1  $\gamma$  and after 12 days 3.0. The corresponding values for the same solution at room temperature were 2.9 and 1.9  $\gamma$ . The more concentrated solutions showed a very gradual rate of decrease in activity under these conditions. After 6 weeks the activity of the 0.1 mg. solution was 5.0  $\gamma$  at ice box and room temperatures, while the 1 mg. solution gave values of 5.8 and 5.3. At 38°, however, the reduction of activity was much more rapid, the 0.1 mg. solution dropping to 4.0  $\gamma$  in 5 days and the 1 mg. solution to 3.6 in 20 days. A concentrated solution of "antibiotin" kindly sent to us by Dr. Woolley for comparison with our material maintained its activity under refrigeration for the testing period of 3 weeks, but after 5 days at 38° the solution lost 50 per cent of its activity and after 12 days 65 per cent. A 1:1000 dilution of the solution was reduced in activity by 50 per cent after 2 days in the ice box. Difference in concentration of the materials tested probably accounts for the disparity in his findings and our earlier observations. Egg white itself maintains its activity over long periods of time, and even 1:100 dilutions of egg white whose avidin concentrations were of the same order as that of the weakest avidin solution tested showed almost no decrease in activity after 48 hours at 38°.

*Effect of Visible Light*—Because of the high content of riboflavin in egg white it was decided to test the effect of added riboflavin to purified preparations of avidin. Avidin and egg white solutions which contained 2.5  $\gamma$  of riboflavin per cc. were therefore tested in the same manner as before, without significant difference in the results. In the avidin assay, however, certain results were somewhat out of line. At the highest levels tested, where the riboflavin concentration during the assay was greater than 0.08  $\gamma$  per cc., an inhibitory effect on avidin activity was observed, which was not substantiated at the lower levels tested. A similar effect of fluorescent materials on toxins in the dark has been noted by some workers (7), which suggested that as fluorescent materials will accelerate inactivation of toxins exposed to light so riboflavin may act as a sensitizer in such a reaction of avidin.

To test this hypothesis solutions of egg white and of avidin were prepared with and without added riboflavin. These solutions were put in glass tubes 5 mm. in diameter, which were placed in a condenser cooled with

running water, and were exposed to a 300 watt bulb at a distance of 12 cm. Control samples were kept at the same temperature in the dark. The examples given in Table I are typical of the results obtained. These values show that avidin is inactivated by irradiation with visible light and that riboflavin sensitizes the reaction. Not only was free avidin so affected as

TABLE I  
*Effect of Visible Light on Avidin Activity*

Material tested	Period of irradiation	Biotin binding power of solutions			
		Without riboflavin		With riboflavin	
		Control	Irradiated sample	Control	Irradiated sample
	hrs	$\gamma$ per cc	$\gamma$ per cc	$\gamma$ per cc	$\gamma$ per cc
Egg White I (1:10 dilution)	24	0 080	0 075	0 080	0 065
" " II (1:100 " )	24	0 0065	0 005	0 006	0 002
Avidin preparation (0 4 mg per cc.)	24	0 77	0 70	0 82	0 075
" " (0 004 mg per cc )	48	0 0075	0 0031	0 0085	0 0*

\* 65 per cent of the biotin originally bound to the avidin was released

TABLE II  
*Effect of Acidity on Avidin Activity*

Source of avidin	Neutral solution, biotin binding capacity	Acid solution (pH 1 8)	
		Biotin added	Remaining biotin-binding capacity
	$\gamma$ per cc	$\gamma$ per cc	$\gamma$ per cc
Egg white	0 89	0 0	0 03
		0 2	0 13
		0 4	0 17
		0 6	0 17
		0 8	0 13
		0 0	0 12
Avidin concentrate (1 cc. = 0 15 mg avidin)	0 51	0 2	0 18
		0 4	0 10

to be incapable of combination with biotin, but in some cases biotin already bound was released. Avidin in egg white is inactivated only a little less readily than avidin in purified preparations. Solutions with a low content of avidin are more easily acted upon than are those of higher concentration.

*Effect of Acidity*—To determine the effect of acidity on its avidin activity, egg white was diluted 1:10 in saline solution and the pH adjusted to the desired level with HCl. Avidin preparations were treated in the same way

in such dilution as to give an activity about the same as that of the egg white solutions. In a preliminary experiment a sample of egg white was found to have lost in 4 hours 70 per cent of its activity at pH 2.6 and 87 per cent at pH 1.8. Other samples showed that as little as 4 per cent of the activity remained at pH 1.8, with no further loss at pH 1.2. In no case was all the avidin activity gone. Avidin solutions differed from solutions of egg white only in occasionally requiring more time for inactivation, probably because in the concentrations used avidin was not completely soluble but remained partly as a fine suspension. When solutions of egg white or of avidin were almost saturated with biotin before acidification, no free biotin was found on assay of unsteamed samples but all of it could be recovered after steaming, showing that the avidin-biotin compound was not affected by the acid treatment. In Table II are given the results of two of a series of experiments made on avidin and egg white to which various amounts of biotin were added before acidification. It may be noted that not only is the avidin-biotin complex not broken down, but its presence seems to afford some protection to the remaining avidin. Not only a larger percentage of the free avidin but a larger amount of it remains active than when no biotin has been added. None of the biotin was dialyzable; therefore no small active fraction of the avidin molecule was split off by the treatment with acid.

#### DISCUSSION

The mechanism or mechanisms of inactivation of avidin cannot be explained on the basis of present information. The effect of the moderate temperatures employed in this study may well be different from the effect of steaming which has long been known to inactivate avidin. The degeneration under the conditions used would lend support to a theory of enzymatic action, but the irradiation data indicate that some other type of reaction is more probable.

The results of the acidity experiments did not confirm the findings of Woolley and Longworth (5) but agreed rather with the earlier work of Parsons and Kelly (6). An explanation of the fact that avidin is not inactivated in the stomach of rats fed diets high in egg white may be found in the different response of avidin and the avidin-biotin complex to treatment with acid. If a considerable portion of the biotin in the diet is available for combination with avidin before the acid can act, the amount released later in the process of digestion and that formed by bacterial action may be insufficient for the needs of the animal. How the avidin-biotin complex resists the digestive action of enzymes must be further investigated.

## SUMMARY

The effect of various factors on the stability of avidin was studied.

1. Solutions of purified avidin lose activity on standing, more readily in dilute than in concentrated solution. In any concentration the rate of destruction is much greater at 38° than at ice box temperature, and except in the most concentrated solutions even room temperature will cause considerable acceleration of the rate. Dilute solutions of egg white are much less affected over this temperature range.

2. Irradiation with visible light inactivates avidin in solutions of egg white or of purified concentrates, the latter more easily. Biotin bound to the avidin may be released. The presence of riboflavin accelerates the action of visible light.

3. When solutions of egg white or of avidin concentrates were brought to pH 1.8 with HCl, their avidin activity was almost but never quite completely destroyed. Biotin already bound to the avidin was not released by the treatment with acid, nor was added biotin, even when the avidin had been almost completely saturated with it.

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# EFFECT OF DIETARY CHOLINE, ETHANOLAMINE, SERINE, CYSTINE, HOMOCYSTEINE, AND GUANIDOACETIC ACID ON THE LIVER LIPIDS OF RATS\*

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Perhaps the most striking characteristic of animals fed a diet poor in lipotropic agents is the finding, recently emphasized (1), that in their bodies and organs large amounts of choline are found in spite of the fact that the pathological changes that have occurred are readily cured by the addition of choline to the diet. Doubtless related to this is the finding reported from this laboratory that the synthesis of choline from its precursors proceeds unimpaired in the bodies of rats even when lipotropic methyl is withheld from the diet (2). These findings, which appear to differentiate choline deficiency from certain other dietary deficiencies, have led us to a reinvestigation of the composition of the lipids that accumulate in the liver under various conditions of diet.

Fatty liver has been produced by restriction of lipotropic methyl (3), and by addition of cystine (4) or homocystine (5) to the diet. In previous studies, the nature and amount of the phosphatide fraction of the liver lipids in fatty livers have been investigated by determination of the fat-soluble choline (6). In the present study, the liver lipid on various levels of choline nutrition has been investigated and parallel studies have been made on animals which received an otherwise adequate diet supplemented with various known or suspected antilipotropic substances. The compounds used were  $\beta$ -ethanolamine, *l*-serine,<sup>1</sup> *l*-cystine, *dl*-homocystine,<sup>2</sup> and guanidoacetic acid. Of these, the last three produced markedly fatty livers. The lipids isolated from the livers of each group of animals were analyzed not only for choline (computed as choline N) but also for total N (Kjeldahl) and P. These analytical data, together with the derived figures for N:P (atomic) ratios, percentages of choline N in the total N of the liver lipid, and weights of choline N and lipid P per liver, are given in Table I.

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<sup>1</sup> The *l*-serine employed was supplied through the generosity of Dr. M. Bergmann of the Rockefeller Institute, to whom the authors are deeply grateful.

<sup>2</sup> The *dl*-homocystine was supplied through the generosity of Dr. E. Borek of the College of Physicians and Surgeons, to whom the authors are deeply grateful.

TABLE I  
Relationship of Various Dietary Supplements to Amount and Composition of Liver Lipids

Liver Lipids

Diet		Mean daily food intake	Initial weight	Final weight	Liver, wet weight	Liver lipids	Liver lipids						
Choline	Supplement						P	Total N	Choline N	N:P	Choline N		P
mg per kg.	mg per kg.	gm.	gm.	gm.	per cent body weight	per cent liver weight	per cent	per cent	per cent		per cent total N	mg. per liver	mg. per liver
0.0	None	3.5	34.4	46.5	7.2	10.3	0.36	0.278	0.030	1.73	10.8	0.108	1.42
5.4	"	3.8	32.4	48.8	5.6	4.0	1.28	0.755	0.230	1.31	30.4	0.246	1.87
					±1.5*	±3.3*							
35.8	"	3.1	32.6	44.1	5.3	3.1	1.63	1.083	0.282	1.47	26.0	0.208	1.17
					±0.6	±0.2							
5.4	Ethanolamine 4.1	2.8	33.3	42.9	6.1	5.9	1.56	0.733	0.181	1.04	24.6	0.278	2.40
					±0.3	±1.1							
5.4	Ethanolamine 12.3	4.5	37.6	52.7	6.0	4.5	1.25	0.645	0.225	1.14	34.9	0.325	1.80
					±0.5	±0.8							
5.4	L-Serine 4.1	3.5	36.8	51.2	5.5	5.0	1.32	0.668	0.206	1.12	30.8	0.286	1.76
					±0.6	±0.9							
5.4	L-Cystine 2.05	3.5	39.2	60.1	6.7	10.7	0.78	0.408	0.069	1.16	17.0	0.305	3.36
					±1.2	±1.6							
5.4	" 6.2	4.4	43.6	63.9	7.0	9.1	0.73	0.353	0.087	1.05	24.7	0.360	3.00
					±0.6	±1.3							
5.4	dl-Homocysteine 4.1	3.3	34.6	49.1	6.7	10.3	0.76	0.389	0.112	1.13	28.8	0.409	2.43
					±1.1	±2.4							
5.4	Guanidoacetic acid 4.1	4.8	38.0	56.1	6.6	13.7	0.53	0.283	0.056	1.18	19.6	0.281	2.68
					±0.4	±3.4							
5.4	Guanidoacetic acid 5.3	3.2	35.2	43.4	6.3	15.9	0.39	0.241	0.013	1.36	4.3	0.057	1.72
					±0.3	±2.5							
5.4	Guanidoacetic acid 8.3	3.1	35.0	51.0	6.1	16.7	0.33	0.159	0.001	1.06	0.8	0.005	1.74
					±1.0	±1.6							

\* Standard deviation =  $\sqrt{\Sigma d^2/n}$ .

#### EXPERIMENTAL

Five male rats were used in each experiment. Newly weaned, 21 day-old litter mates were selected and kept together in one cage which was lined with wood shavings and cleaned daily. Diet and water were given *ad libitum* but records were kept of mean daily food consumption. In all cases the rats grew and gained in weight, and there were no deaths during the course of feeding which lasted 10 days.

The basal diet employed was that of Griffith and Wade (7) and comprised

15 parts of extracted casein, 35 parts of lard, 4 parts of salt mixture (8), 1 part of  $\text{CaCO}_3$ , 2 parts of agar, 5 parts of cod liver oil, 6 parts of yeast, and 32 parts of sucrose.<sup>3</sup> The dry diet was intimately mixed with the various supplements (Table I).

After 10 days of feeding, the animals were killed by ether and final body weights taken. Liver, kidneys, spleen, and thymus were dissected out and weighed individually. The weights of only the livers are given in Table I; with the other organs no significant changes were noted. The livers were individually extracted with  $\text{CHCl}_3$ , according to the method of Channon, Platt, and Smith (9), the primary extraction being continued for 12 hours. The resulting  $\text{CHCl}_3$  solutions were taken to dryness, first under a bell jar in a stream of  $\text{N}_2$ , and then in an oven at  $110^\circ$  for 12 hours. The weights of the lipids are tabulated as percentages of wet weight of liver.

The five samples of liver lipids from each feeding experiment were pooled in toluene solution, and this was extracted in a separatory funnel with 1 per cent  $\text{HCl}$  to remove non-lipid nitrogenous contaminants (10). In some cases centrifugation was necessary to break the emulsions that resulted. The washed toluene solutions were made up to a volume of 100 cc. and these solutions were used for the following analytical procedures.

The total lipid content of each solution was computed from the residue weight of a 2 cc. aliquot after the removal of the solvent in an oven at  $110^\circ$ . 2 cc. aliquots were concentrated in digestion tubes for gravimetric micro phosphorus determinations which were carried out by Mr. W. Sasehek. 10 cc. aliquots were freed of toluene by steam distillation and digested for routine micro-Kjeldahl N determinations. Aliquots of 10 to 20 cc. were evaporated to dryness and choline determined essentially according to Jacobi, Baumann, and Meek (6). The isolated choline reineckate, made up to 10 cc. in acetone solution, was read in a Coleman universal spectrophotometer at  $530 \text{ m}\mu$ , which was shown to be the absorption maximum of this compound. The instrument had previously been calibrated by taking readings on authentic solutions of choline reineckate at various concentrations in the range used.

#### DISCUSSION

From the first three experiments, which differed from one another only in the level of choline nutrition, it is apparent that the bulk of the lipid deposited in the liver in choline deficiency is not phosphatide. In accord

<sup>3</sup> Qualitatively this diet gave similar results in our experiments as in those of Griffith and Wade (7). Quantitatively, however, we obtained a consistently milder degree of fatty infiltration of the liver than did these authors. The explanation for this discrepancy may lie in a difference in the quality of yeast or casein used in the two laboratories.



product of the former, methionine, can and does serve in turn as a methyl donor (19).

In addition to producing marked alterations in the liver, the feeding of guanidoacetic acid also brought about changes in the kidney. These changes were not grossly manifest in the animals receiving the lowest dosage of guanidoacetic acid, but on the two higher levels of feeding hemorrhagic lesions were noted in 60 and 90 per cent respectively of the kidneys examined.

#### SUMMARY

The quantity of liver lipid and its composition with respect to nitrogen, phosphorus, and choline have been investigated in rats on various levels of choline nutrition and with addition to the diet of ethanolamine, serine, cystine, homocysteine, and guanidoacetic acid.

The liver fat which results from dietary choline deficiency is poor in lecithin, whereas that which results from the feeding of cystine or homocysteine is abnormally rich in this phosphatide. Ethanolamine and serine, when fed, produce no great increase in liver lipid, but bring about a rise in monoamino phosphatides of this organ.

The feeding of guanidoacetic acid causes a marked fatty liver with a coincident striking decrease in the choline content of the liver lipid. A hypothesis is offered relating these latter findings to the well known irreversible biological methylation of guanidoacetic acid.

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## SOME FACTORS AFFECTING THE SYNTHESIS OF ASCORBIC ACID IN THE ALBINO RAT

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The investigations of Phillips and coworkers (1, 2) demonstrating the beneficial effects of ascorbic acid treatment in certain types of sterility have focused attention on the synthesis of ascorbic acid in the animal body. Previous to these investigations it was generally assumed that the synthesis of ascorbic acid in animals other than Primates and guinea pigs was adequate at all times for normal body functions. The finding that many of the animals showing abnormal reproductive performance had lower ascorbic acid concentrations than normally found in certain body fluids prompted the use of the ascorbic acid treatments and cast doubt on this assumption.

Several studies have shown that certain dietary factors affect the rate of ascorbic acid synthesis. Sure, Theis, and Harrelson (3) observed that deficiencies of vitamins A, B<sub>1</sub>, and riboflavin caused a decrease in the ascorbic acid of rat tissues. Frederick, Guerrant, Dutcher, and Knight (4) showed that the amount of ascorbic acid excreted by the rat was influenced by the nature of the carbohydrate in the diet. The same laboratory produced evidence that ascorbic acid was rapidly destroyed in the bovine rumen and the oral administration of massive dosages failed to increase the concentration in the blood plasma or the amount secreted in the milk and had but slight effect on the amount excreted in the urine (5). On the other hand, Bortree, Scheidenhelm, and Huffman (6) have reported that the oral administration of trichlorobutyl alcohol caused a marked increase in concentration of ascorbic acid in the blood plasma of the bovine. Similar results (unpublished) have been obtained in this laboratory. Earlier work by Musulin *et al.* (7) with rats supports the idea that dietary factors influence the rate of ascorbic acid synthesis in the animal body. Phillips and coworkers (8) have observed that low ascorbic acid values for blood plasma of calves were increased by feeding shark liver oil rich in vitamin A. Since vitamin A deficiency frequently occurs among farm animals under current systems of feeding and management, additional fundamental data on this subject seem desirable.

The laboratory rat is known to synthesize ascorbic acid in its body and

was therefore selected as the experimental animal for the investigations reported herein because of the simplicity of regulating the diet and controlling other environmental factors.

#### EXPERIMENTAL

*Effects of Level of Vitamin A Intake on Blood Plasma Ascorbic Acid Level and Rate of Ascorbic Acid Excretion*—For the study of the effects of the level of vitamin A intake on the ascorbic acid level of the blood plasma, three groups of weanling, male albino rats were maintained on the U.S.P. vitamin A-deficient ration supplemented with graded dosages of vitamin A from standard reference oil. The amount administered was maintained at levels of 10, 20, and 30 international units per kilo of body weight daily (Groups I, II, and III, respectively).

After approximately 65 days on this ration, the experiment was terminated. The animals were killed by exsanguination following preliminary ether anesthetization and the blood was collected for determinations of the plasma ascorbic acid. Ascorbic acid was determined by the procedure of Mindlin and Butler (9) by the titration technique. Results of this study are presented in Table I. It will be noted that limiting the vitamin A intake resulted in a significant decrease in the blood plasma ascorbic acid. Since completion of this work, determinations of blood plasma ascorbic acid on a few triplicates of animals receiving similar dietary treatment were made with the Evelyn photoelectric colorimeter. Although the ascorbic acid concentrations found were somewhat lower than those obtained by the titration technique, the same order of difference between groups was substantiated.

These data on blood plasma ascorbic acid are supported by studies of ascorbic acid in the urine. For this study (Experiment I) twelve weanling albino rats were divided into two comparable groups. One group received the U.S.P. vitamin A-free ration and other group the same ration supplemented with 90 I.U. of vitamin A from standard reference oil daily. At about the time the effects of the deficiency began to cause a decrease in the rate of growth in the unsupplemented group, urine collections were made every other day for a period of 2 weeks. During the collections three animals were placed in each collection cage. The urine was preserved during the 24 hour collection periods according to the method of Longenecker *et al.* (10) and the ascorbic acid determined by an adaptation of the macro photometer method of Mindlin and Butler (9). These data are summarized in Table II, Experiment I.

What appeared to be a sex difference in the rate of ascorbic acid excretion was noted in some of our preliminary work. To test this, a second experiment was conducted in which the males and females were grouped

and segregated in such a way that there was equal sex and litter distribution. One animal was placed in each collection cage. The animals used in this experiment were approximately 30 days of age when started and the

TABLE I

*Effect of Level of Vitamin A Intake upon Blood Plasma Ascorbic Acid in Rats*

Rat No.	Litter No.	Days on experiment	Vitamin A supplement per kilo body weight	Ascorbic acid per 100 cc. blood plasma
			<i>international units</i>	<i>mg.</i>
E-1667A	1	63	10	0.74
E-1668A	1	63	20	Lost
E-1669A	1	63	30	1.04
E-1670A	2	62	10	0.521
E-1672A	2	62	20	0.856
E-1671A	2	62	30	1.04
E-1673A	3	53	10	0.585
E-1674A	3	53	20	0.741
E-1675A	3	53	30	0.702
E-1676A	4	70	10	0.660
E-1677A	4	70	20	0.746
E-1678A	4	70	30	0.819
E-1679A	5	77	10	0.552
E-1680A	5	77	20	0.762
E-1681A	5	77	30	0.864
E-1701	6	62	10	0.742
E-1702	6	62	20	0.952
E-1703	6	62	30	1.056
E-1710	8	61	20	0.780
E-1711	8	61	30	0.983
E-1712	9	71	10	0.716
E-1713	9	71	20	0.739
E-1714	9	71	30	1.010
E-1715	10	73	10	0.384
E-1716	10	73	20	0.576
E-1717	10	73	30	0.720
E-1721	11	71	10	0.384
E-1722	11	71	20	0.576
E-1723	11	71	30	0.720
Average, 9 rats, Group I....		65.8	10	0.5877
" 9 " " II..		65.8	20	0.7375
" 10 " " III..		65.8	30	0.8954

dietary treatment was similar to that of Experiment I. Urine collections were started immediately and continued at about weekly intervals throughout the duration of the experiment. The experiment was terminated

after 55 days and a total of nine 24 hour collections was made and analyzed. These data are summarized in Table II, Experiment II.

It will be noted from these data that the excretion of ascorbic acid is less in the animals receiving the unsupplemented vitamin A-free ration. These differences are greater toward the end of the experimental period, as demonstrated by the data of Experiment I. An average of the last five tests of Experiment II also demonstrates this. Average daily excretions of ascorbic acid for the last five tests of Experiment II were vitamin A-deficient males, 0.064 mg.; vitamin A-supplemented males, 0.108 mg.; vitamin A-deficient females, 0.040 mg.; vitamin A-supplemented females, 0.096 mg.

It may also be noted that as time elapsed on the supplemented u.s.p. vitamin A-free diet, the rate of ascorbic acid excretion declined. Other positive evidence of this was obtained by collecting and assaying the urine

TABLE II  
*Effects of Vitamin A Deficiency on Ascorbic Acid Excretion*

Experiment No.	No. of animals	No. of tests	Ascorbic acid excreted daily (average)	
			Vitamin A-free ration	Same ration + 90 l. u. vitamin A
			mg.	mg.
I	6*	8	0.034	0.099
II	2♂	9	0.182	0.226
	2♀	9	0.039	0.123

\* Equal sex and litter distribution.

of animals of the same age which had been maintained on the regular stock ration used in our laboratory. These invariably gave higher results than were found for the animals on the supplemented u.s.p. ration. This would indicate that there are dietary factors other than vitamin A which are operative in maintaining a normal rate of ascorbic acid synthesis and excretion.

These experiments support the blood plasma studies in demonstrating the effects of vitamin A intake on ascorbic acid synthesis. While we recognize that there are possibly other dietary factors which influence the rate of ascorbic acid synthesis in the animal body, these results are of interest in an assessment of the rations frequently being fed to breeding animals of both sexes. Our observations have been that bulls with low blood ascorbic acid and poor breeding performance are usually being fed poor quality roughage. We have also studied the ascorbic acid concentration of cows in herds being fed excellent and poor quality roughage. The

average ascorbic acid level of blood plasma was lower in the herd receiving the poor quality roughage.

*Effects of Synthetic Estrogenic Hormone (Diethylstilbestrol) on Ascorbic Acid Excretion*—Phillips *et al.* (2) have reported data showing an increase in blood plasma ascorbic acid during estrus in the cow. These data have been substantiated by similar unpublished data of this laboratory and prompted a study of the effects of the estrogenic hormone on the urinary excretion of ascorbic acid. The estrogenic hormone employed was diethylstilbestrol.<sup>1</sup> Sixteen female rats of approximately the same age were selected and allotted to the experiment. Selected pairs were placed in collection cages and preliminary data on ascorbic acid excretion obtained. They were then divided into two groups on the basis of the levels of ascorbic acid excretion and one group was castrated. After a second collection and assay period, the groups were again subdivided on the basis of their ascorbic acid excretion into the groups shown in Table III, Group I, intact controls; Group II, intact animals, stilbestrol-treated; Group III, castrated controls; Group IV, castrated animals, stilbestrol-treated.

The animals selected for the stilbestrol treatment were given 100  $\gamma$  of diethylstilbestrol daily in 0.1 cc. of cottonseed oil solution. The control animals received an equivalent amount of the oil. The oil was measured into the mouth from a tuberculin syringe. During the course of the experiment, the animals were maintained on our regular stock colony ration.

Vaginal smears were made daily throughout the experiment in order to determine the course of the estrous cycle and the degree of stimulation following stilbestrol treatment. The stilbestrol-treated, castrated animals had typical estrus smears throughout the period of stilbestrol treatment. The stilbestrol-treated, intact animals showed atypical estrus smears occasionally during the period of treatment. Other than this the smears were typical of estrus. These atypical smears were characterized by great numbers of polymorphonuclear leucocytes among the keratinized epithelial debris.

The results of the ascorbic acid determinations are summarized in Table III. The stilbestrol treatment resulted in an increase in the rate of ascorbic acid excretion. This increase was much more marked in the castrated animals, indicating that the ovary may exert a neutralizing effect on stilbestrol stimulation.

During the week of stilbestrol treatment, the animals receiving stilbestrol lost an average of 15 gm. in body weight.

The results of this experiment offer a possible explanation for the in-

<sup>1</sup> We wish to thank Merck and Company, Inc., for their courtesy in supplying the diethylstilbestrol used in this study.



creased ascorbic acid concentration in the blood plasma of cows during estrus.

*Site of Ascorbic Acid Synthesis in Animal Body*—Information regarding the site of ascorbic acid synthesis should prove to be of considerable fundamental importance. The concentration of ascorbic acid in certain organs and tissues has been noted. High concentrations have been reported in the pituitary by Phillips and Stare (11) and Glick and Biskind (12) and in the adrenal and reproductive glands by Bessey and King (13), Glick and Biskind (14), Biskind and Glick (15), and Giroud (16). These reports of ascorbic acid concentration in these organs and tissues might be interpreted as indicating storage in these organs or synthesis by them. Evidence that ascorbic acid is still synthesized in the body after pituitary

TABLE III

*Effect of Stilbestrol Treatment on Ascorbic Acid Excretion in Normal and Castrated Female Rats*

Group No. (4 rats each)	Average daily ascorbic acid excretion		
	Precastration, 3 tests	Postcastration, 5 tests	Treatment period, 6 tests
	mg.	mg.	mg.
I. Intact controls.....	0.498	0.476	0.429
II. " animals, stilbestrol-treated.....	0.500	0.399	0.573
III. Castrated controls.....	0.670	0.445	0.586
IV. " animals, stilbestrol-treated.....	0.634	0.417	1.559

removal has been presented by Leblond and Chamorro (17). The studies reported herein substantiate this report by demonstrating synthesis in a different way and provide additional information regarding synthesis in the absence of the gonads and adrenal glands.

The amount of ascorbic acid normally excreted in the urine is quite small and changes resulting from gland removal may not be particularly convincing. It was felt that the finding of Longenecker *et al.* (18) that certain organic compounds greatly increased the rate of synthesis should prove an effective tool in measuring the rate of synthesis in animals operated upon.

The plan of the experiment was briefly as follows: Seven partially grown rats, weight approximately 200 gm., were placed in individual collection cages and fed our standard stock colony ration which consisted of a dry concentrate mixture plus fluid whole milk. 20 mg. of chloretone in a 50 per cent alcohol solution were added to each daily allowance of milk. The animals were kept on this ration throughout the experiment. After the maximum ascorbic acid excretion had been reached, the animals were

castrated. 12 days following castration their pituitary glands were removed and 11 days later their adrenal glands were removed.

Ascorbic acid determinations were made on 24 hour urine samples every other day. Following each operation 2 days elapsed before urine collections were made except following adrenalectomy, when 3 days elapsed before a urine collection. Preliminary work had shown that there was an immediate postoperative decrease in ascorbic acid excretion, which persisted for about 2 days.

The results of this experiment are summarized in Table IV. The feeding of chloretone produces a marked increase in the rate of ascorbic acid excretion. Tests on a large number of animals on our stock colony

TABLE IV  
*Effects of Castration, Hypophysectomy, and Adrenalectomy on Ascorbic Acid Excretion of Chloretone-Fed Rats*

Rat No. and sex	Average daily ascorbic acid excretion			
	Previous to castration, 5 tests	After castration, 5 tests	After castration and hypophysectomy, 5 tests	After castration, hypophysectomy, and adrenalectomy, 3 tests
	mg.	mg.	mg.	mg.
E-1821 ♀	13.56	16.30	5.63 (4 tests)	2.98
E-1822 ♂*	12.79	12.28	9.22	6.12
E-1823 ♂	11.59	11.13	6.92	2.10
E-1824 ♂*	13.46	9.61	9.97	5.71
E-1825 ♀	12.37	13.82	10.39	6.21
E-1826 ♀	11.72	13.90	5.15	3.50
E-1827 ♀	10.99	14.09		

\* Hypophysectomy incomplete (approximately one-half of the gland was removed).

ration show average daily ascorbic acid excretions of slightly less than 1.0 mg. daily. The data summarized in Table IV are for the five tests preceding castration only. Some of the animals had been receiving chloretone for over a month, without diminution in rate of ascorbic acid excretion. Others had been receiving the drug for a much shorter time. We can safely assume, therefore, that the decreases in excretion during the course of the experiment following the operations were not a result of a refractory condition induced by continued chloretone treatment.

The effects of castration are probably insignificant in so far as ascorbic acid synthesis is concerned. Certainly there was no decrease in the rate of synthesis. Following hypophysectomy there is a decrease in the rate of synthesis; also there is a further decrease following adrenalectomy. Yet ascorbic acid is still synthesized and the decreases noted may be no

greater than might be expected from the decrease in metabolic rate following hypophysectomy and the general moribund condition following adrenalectomy when the salt intake is *not* increased.

We are inclined to believe that the synthesis of ascorbic acid is not a specific function of any single gland or tissue but more probably a general metabolic function. *In vitro* synthesis by liver, kidney, and spleen tissue has been reported by Guha and Ghosh (19) and further evidence of ascorbic acid synthesis by rat tissues has been obtained by the *in vitro* studies of Smythe and King (20). Furthermore, the concentrations found in certain glands and tissues are indicative of storage rather than synthesis.

#### SUMMARY

The effect of the vitamin A intake on ascorbic acid synthesis was studied by determining the level of ascorbic acid in the blood of animals which had received graded daily dosages of vitamin A and the urinary excretion of ascorbic acid by animals receiving the U.S.P. vitamin A-free diet with and without vitamin A supplement.

A suboptimum vitamin A intake resulted in a decreased concentration of blood plasma ascorbic acid and a decrease in urinary ascorbic acid.

The effect of the estrogenic hormone on ascorbic acid synthesis was studied by feeding synthetic estrogenic hormone (stilbestrol) to normal and castrated female rats and determining the rate of excretion of ascorbic acid. The synthetic estrogenic hormone administered at the rate of 100  $\gamma$  daily resulted in an increased excretion of ascorbic acid in the intact and castrated female. The response was slightly greater in the case of the castrated females. These results offer a possible explanation for the rise in blood plasma ascorbic acid during estrus in the cow.

The high concentration of ascorbic acid in the ovary (corpus luteum), pituitary, and adrenal glands may be interpreted as indicating either storage or synthesis by these tissues. In an attempt to throw further light on this subject, these glands were removed from a group of chloretone-fed female rats and the effects on ascorbic acid excretion determined. Chloretone-fed animals with gonads, pituitary, and adrenal glands removed were still capable of synthesizing ascorbic acid. The rate of synthesis declined following hypophysectomy and again following adrenalectomy. This decline was probably no greater than might be expected in consideration of the general debilitating effect of these operations. This is interpreted to mean that no particular organ or gland is involved but that ascorbic acid synthesis is a general metabolic function.

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# THE INVESTIGATION OF AMINO ACID REACTIONS BY METHODS OF NON-AQUEOUS TITRIMETRY

## I. ACETYLATION AND FORMYLATION OF AMINO GROUPS\*

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This paper is primarily concerned with the formation of N-acetyl and N-formyl derivatives of amino acids by acetic anhydride and formic acid in non-aqueous medium.

The method of acetylation used, suggested by the work of Knoop and Blanco (12), consists of permitting acetic anhydride to react with the amino acid in a medium of glacial acetic acid. The investigation of Bergmann and Zervas (4) indicates that under these conditions racemization is a minor factor provided that no excess of acetic anhydride be left or that the reaction is not permitted to continue beyond the time necessary for completion of the acetylation stage. Easy isolation of the reaction products, because only readily volatilized reagents are employed, is another advantage of the procedure of Knoop and Blanco.

However, the reaction has been applied only to a few amino acids and only at elevated temperatures. Existing data (*cf.* Raiford *et al.* (16)) show clearly that the reactivity of amino groups with acetic anhydride differs widely with different amino compounds. An exploration of the rates of reaction between acetic anhydride and various amino acids in acetic acid at room temperature is here reported. Since amino acids, but not their acyl derivatives, act as bases toward acetic perchloric acid (*cf.* Toennies and Callan (19)), this reagent provides a convenient means for directly following the course of N-acylation. Besides acetylation we have studied formylation by this method and under similar conditions, based upon the work of Fruton and Clarke (8), Billmann *et al.* (5), and of du Vigneaud *et al.* (21-23), which demonstrated the formation of pure formyl derivatives by the action of formic acid and acetic anhydride at room temperature.

Primary aims of the experimental work were to see to what extent significant differences might exist between different types of amino acids with regard to their acetylation and formylation rates, to what extent the distal basic groups of the diamino acids participate in these reactions, and to what extent acetylation and formylation differ in these two regards.

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### *Titrations*

With all amino acids investigated it was consistently observed that on acetylation and formylation the perchloric acid consumption continuously decreased, but never completely reached the blank value. Furthermore, while the total titration value decreased, the interval between the green and the yellow shade showed marked increases, which in all cases were substantially greater in acetylations than in formylations. That these phenomena are characteristic of the respective acyl derivatives themselves is indicated by the behavior of pure acetyl- and formyl-*dl*-alanine (their preparation is described below) under comparable conditions. 0.25 mm of acetylalanine dissolved in 5 cc. of acetic acid was titrated with 0.1 N  $\text{HClO}_4$  in acetic acid (Toennies and Callan (19)). Titration to the green shade of crystal-violet required 0.13 cc., to a yellow shade 0.55 cc. 0.25 mm of formylalanine under the same conditions required 0.08 and 0.16 cc. Similar values resulted when the medium consisted of 10 cc. of acetic acid and 0.4 cc. of 98 per cent formic acid. In these titrations the yellow shade is an approximation of, but not identical with, the pure golden yellow of blank titrations. Apparently the residual basicity of the nitrogen is distinctly greater in the case of acetyl substitution than in the case of formyl substitution. In the kinetic experiments no attempt was made to correct for the uncertainty introduced by the residual titration values of the reaction products, and all calculations (Figs. 1 and 2) are based on the uncorrected green end-points.

### *Acetylations*

A solution of the amino acid (0.025 to 0.10 M) in glacial acetic acid was prepared. Heat was used for this step if necessary; subsequent perchloric acid titration showed in no case evidence of acetylation by the warm acetic acid. The solutions were combined, in 50 or 100 cc. volumetric flasks, with a definite amount of concentrated acetic anhydride (about 10 M<sup>1</sup>) and made up to volume at room temperature (24–27°) with acetic acid. 5 cc. or larger aliquots were titrated at intervals with acetic perchloric acid. Initial uncertainty about the exact amount of water present in the acetic acid and about the rate of its reaction with acetic anhydride led to the adoption of a fairly high ratio (mostly between 7 and 8) of acetic anhydride to basic nitrogen. Later the water content of the acetic acid was found<sup>2</sup> to be only 0.11 M and it was also established

<sup>1</sup> The exact value was determined according to Toennies and Elliott (20).

<sup>2</sup> By permitting the water to react with acetic anhydride in the presence of perchloric acid (Toennies and Elliott (20)) and measuring the excess acetic anhydride by letting it react with anthranilic acid and determining the unacetylated excess of the latter by titration with acetic perchloric acid (*cf.* Kilpi (11)).

that its reaction with acetic anhydride under the experimental conditions is extremely slow. Fig. 1 shows the results of the acetylations which were followed in solution. In all cases the free amino acids were used; in the case of lysine the latter was readily obtained in solution by agitating for  $\frac{1}{2}$  hour a mixture of equivalent amounts of lysine dihydrochloride and silver acetate, suspended in acetic acid. The data in Fig. 1 indicate no major differences in acetylation rates among the neutral amino acids.

The known instability of *tryptophane* toward acid is also in evidence in the non-aqueous system, as samples of the reaction mixture after titration with perchloric acid soon turn brown on standing. The two experiments

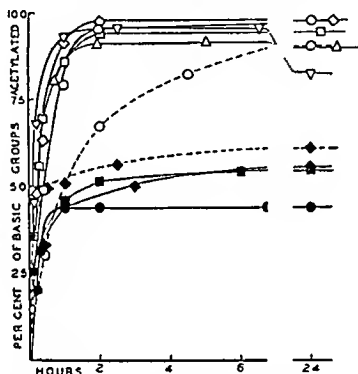


FIG. 1. The reaction of amino acids with acetic anhydride in acetic acid at room temperature (24–27°), observed by titration of basic groups with acetic perchloric acid.  $H_2O$  present 0.11 M. ○ (solid line) *dl*-alanine 0.095 M,  $(CH_3CO)_2O$  0.41 M; (dash line) *dl*-alanine 0.102 M,  $(CH_3CO)_2O$  0.15 M; □ *l*-leucine 0.050 M,  $(CH_3CO)_2O$  0.36 M; △ *l*-tryptophane 0.049 M,  $(CH_3CO)_2O$  0.36 M; ▽ *l*-cysteine 0.052 M,  $(CH_3CO)_2O$  0.40 M; ◇ *l*-hydroxyproline 0.050 M,  $(CH_3CO)_2O$  0.36 M; ● *l*-histidine 0.048 M,  $(CH_3CO)_2O$  0.42 M; ■ *l*-arginine 0.023 M,  $(CH_3CO)_2O$  0.37 M; ◆ (solid line) *l*-lysine 0.025 M,  $(CH_3CO)_2O$  0.40 M; (dash line) *l*-lysine 0.025 M,  $(CH_3CO)_2O$  0.81 M.

with *alanine* and *lysine* each show that acetylation takes place less rapidly with smaller excesses of acetic anhydride.

The behavior of one of the basic groups of the *diamino acids* is so similar to that of the neutral amino acids that it seems logical to attribute the reaction to the  $\alpha$ -amino group, which is less basic than the distal one (Edsall (6)). The reaction curves show that additional basic groups are acetylated only slowly, if at all, probably because they exist preponderantly in the cationic form. In earlier studies on the three diamino acids (Gerngross (9), Bergmann and Köster (3), Neuberger (14), Synge (18)) similar results were recorded; only in the case of *lysine* is there some evidence for the partial formation of a diacetyl derivative (Neuberger (14), Synge (18)).



The case of *cysteine* requires special mention, because in its acetylation the perchloric acid titration value passes through the normal minimum value, only to increase again. After 45 hours the curve had gone down to the 75 per cent level. The nature of this anomaly has not at present been investigated.<sup>3</sup> The same phenomenon, to a smaller but definite extent, occurs in the formylation of *cysteine*, but not in the case of *dl-serine*. With this, a 0.05 M solution could not be obtained; however, after shaking overnight with 8 moles of *acetic anhydride*, solution was complete and titration indicated 97 per cent N-acetylation.

That the present experimental conditions do not favor the acetylation of hydroxy groups is suggested by the isolation of pure *N-acetylhydroxyproline*.

*l-Glutamic* and *l-aspartic* acid required 1 or 2 days of heating on the steam bath before dissolving completely (1 mm of amino acid, 7 mm of *acetic anhydride*, 20 cc. of *acetic acid*). The titration values then corresponded to complete acetylation. A similar suspension of *l-cystine* showed much evidence of decomposition after 1 week of heating, and most of the crystals remained undissolved.

In order to examine the rôle of the ionic state of the amino acid in the reaction with *acetic anhydride*, 10 mm of *dl-alanine* were dissolved by means of *acetic acid* and 13.1 mm of  $\text{HClO}_4$ . 70.5 mm of *acetic anhydride* were added and the solution was diluted to 100 cc. with *acetic acid*. The total amount of  $\text{H}_2\text{O}$  present was 45 mm. The excess of free  $\text{HClO}_4$ , corresponding to 3.1 mm, should (Toennies and Elliott (20)) induce rapid disappearance of water by reaction with *acetic anhydride*. Titration of samples of the solution with a 0.1 N solution of anhydrous sodium acetate in *acetic acid*, to the green end-point of crystal-violet, gave the following values (the calculated zero value is 3.1 mm): after 20 minutes 3.2 mm, after 20 hours 3.4 mm, after 2 weeks 4.2 mm, after 4 weeks 5.1 mm, after 6 weeks 5.8 mm, and after 16 weeks 8.3 mm. It is evident that amino acetylation under these conditions is several thousand times slower than in the absence of  $\text{HClO}_4$  (Fig. 1). Przylecki *et al.* (15), Hammett (10), and Edsall (6) have adduced evidence indicating that in *acetic acid* the uncharged form of the amino acid,  $\text{R}(\text{COOH})\text{NH}_2$ , predominates over the zwitter ionic form. The present observations support the view that a free electron pair on the uncharged nitrogen atom represents the point of attack in the acetylation, which in *acetic acid* solution can readily be effected unless the cationic form is induced by the presence of strong acid.

<sup>3</sup> Possibly the conditions of the *acetic acid* medium favor a migration of the acetyl from N to S (by way of a thiazoline ring as an intermediate) analogous to the shift of benzoyl from N to O observed by Bergmann *et al.* (2) in *aqueous media*.

## Formylations

The results of our exploratory experiments on formylation rates are summarized in Fig. 2. In these experiments the amino acid was dissolved in 98 per cent formic acid, some acetic acid and the desired amount of acetic anhydride were added, and the volume was completed with acetic acid. 1 cc. samples were titrated after dilution with 10 cc. of acetic acid. The use of higher amino acid concentrations in the formylations than in the acetylations was resorted to in order to avoid the disturbing effect of larger amounts of formic acid (Toennies and Callan (19)) in the titrations.

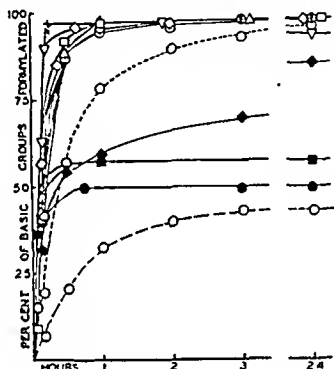


FIG. 2. The reaction of amino acids with formic acid and acetic anhydride at room temperature (24–27°), observed by titration of basic groups with acetic per-chloric acid. ○ (solid line) *dl*-alanine 0.245 M,  $(\text{CH}_3\text{CO})_2\text{O}$  0.91 M,  $\text{HCOOH}$  10.4 M,  $\text{H}_2\text{O}$  0.50 M; (dotted line) *dl*-alanine 0.245 M,  $(\text{CH}_3\text{CO})_2\text{O}$  0.40 M,  $\text{HCOOH}$  10.4 M,  $\text{H}_2\text{O}$  0.50 M; (dash line) *dl*-alanine 0.245 M,  $(\text{CH}_3\text{CO})_2\text{O}$  0.10 M,  $\text{HCOOH}$  10.4 M,  $\text{H}_2\text{O}$  0.50 M; □ *dl*-serine 0.258 M,  $(\text{CH}_3\text{CO})_2\text{O}$  0.91 M,  $\text{HCOOH}$  10.4 M,  $\text{H}_2\text{O}$  0.50 M; △ *l*-tryptophane 0.247 M,  $(\text{CH}_3\text{CO})_2\text{O}$  1.11 M,  $\text{HCOOH}$  10.4 M,  $\text{H}_2\text{O}$  0.50 M; ▽ *l*-cysteine 0.257 M,  $(\text{CH}_3\text{CO})_2\text{O}$  1.11 M,  $\text{HCOOH}$  10.4 M,  $\text{H}_2\text{O}$  0.50 M; ◇ *l*-tyrosine 0.250 M,  $(\text{CH}_3\text{CO})_2\text{O}$  1.11 M,  $\text{HCOOH}$  10.4 M,  $\text{H}_2\text{O}$  0.50 M; + *l*-aspartic acid 0.260 M,  $(\text{CH}_3\text{CO})_2\text{O}$  0.91 M,  $\text{HCOOH}$  10.4 M,  $\text{H}_2\text{O}$  0.50 M; × *l*-glutamic acid 0.254 M,  $(\text{CH}_3\text{CO})_2\text{O}$  0.91 M,  $\text{HCOOH}$  10.4 M,  $\text{H}_2\text{O}$  0.50 M; ● *l*-histidine 0.260 M,  $(\text{CH}_3\text{CO})_2\text{O}$  1.00 M,  $\text{HCOOH}$  5.2 M,  $\text{H}_2\text{O}$  0.28 M; ■ *l*-arginine 0.253 M,  $(\text{CH}_3\text{CO})_2\text{O}$  0.91 M,  $\text{HCOOH}$  10.4 M,  $\text{H}_2\text{O}$  0.50 M; also (identical curve) *l*-arginine 0.260 M,  $(\text{CH}_3\text{CO})_2\text{O}$  1.11 M,  $\text{HCOOH}$  5.2 M,  $\text{H}_2\text{O}$  0.28 M; ◆ *l*-lysine 0.199 M,  $(\text{CH}_3\text{CO})_2\text{O}$  0.96 M,  $\text{HCOOH}$  9.2 M,  $\text{H}_2\text{O}$  0.43 M.

A comparison of the reaction curves of Figs. 1 and 2 does not reveal any evidence for a major difference in the velocities of acetylation and formylation, if allowance is made for the increased rate of amino acid disappearance which is to be expected as a result of the higher amino acid concentrations in the experiments of Fig. 2. In the latter experiments the

formyl derivative is the exclusive or greatly predominating reaction product. The possibility that formic acid or the mixed formic-acetic anhydride (Béhal (1), Schierz (17), and Billmann *et al.* (5)) is capable of displacing primarily formed N-acetyl groups by formyl groups is susceptible to experimental verification because of the characteristic differences, mentioned above (cf. "Titrations"), between formyl and acetyl derivatives in the behavior at the titration end-point. An experiment in which to 40 cc. of an acetic acid solution of 10 mm of alanine and 20 mm of acetic anhydride after 24 hours 8 cc. of 98 per cent formic acid were added, showed, on titration of samples during several days, no evidence suggesting conversion of the initially formed acetyl derivative to the formyl derivative.

With regard to the behavior of individual amino acids, Fig. 2 shows that just as in the acetylation curves of Fig. 1 no outstanding differences in the rates of formylation of the  $\alpha$ -amino groups appear to exist except that the two dicarboxylic compounds, *aspartic* and *glutamic acids*, show more rapid reactions than any of the other compounds. The instability of *tryptophane* under acid conditions, mentioned in connection with its acetylation, is more prominent in the formylation. The higher acidity of formic acid causes a slow discoloration even in the untitrated solution, and after perchloric acid titration the samples soon turn wine-red. Among the three basic amino acids studied only *lysine* shows clear evidence of a complete reaction on both basic groups. Here, just as in the corresponding acetylations, the second amino group seems to show greatly depressed reactivity, and the same theoretical considerations that were given there are applicable. Formation of *diformyllysine* was confirmed by isolation. The manner of obtaining the lysine solution was similar to that used in the acetylation experiment. The experiment with *histidine* shows clearly that only one basic group is involved. In the case of *arginine* two experiments were run, the chief difference between which is a 100 per cent variation in the amount of formic acid used. The fact that the two curves are indistinguishable in Fig. 2 is in harmony with the hypothesis that the reaction is caused by an active interaction product of acetic anhydride and formic acid the concentration of which is governed by the amount of acetic anhydride when formic acid is present in excess. This view is likewise supported by the three experiments with *alanine* shown in Fig. 2; the lowest of the three curves shows that when formic acid and the amino acid are present in excess the amount of amino acid converted is equal to the amount of acetic anhydride present. Incidentally the evidence of this curve also justifies the conclusion that under the prevailing conditions the extent of the reaction of acetic anhydride with water, although its concentration exceeds that of the anhydride and the amino acids, is negligible.

A question that with others must remain for future investigation con-

cerns the significance of the partial extent of the reaction of the second basic group which is indicated by the formylation curves of arginine and by the acetylation curves of arginine and lysine, and which may suggest the existence of reversible equilibria.

### Isolations

*N-Acetyl-dl-alanine*—25 mm of *dl*-alanine suspended in 60 cc. of acetic acid and 5 cc. of acetic anhydride had dissolved after 90 minutes of shaking. After standing one night, when perchloric acid titration showed nearly complete reaction, the solution was evaporated to dryness in a vacuum desiccator over saturated NaOH. Equivalent weight (phenolphthalein) 133.4, 133.7, yield 100 per cent; after recrystallization from isopropyl acetate (25 cc. per gm., 70 per cent yield) 132.4, 132.9; calculated 131.1. M.p. 135–136° (corrected); previously recorded values (Fischer and Otto (7), Synge (18)) 137.5° (corrected), 136°.

*N-Acetyl-dl-methionine*—150 mm of *dl*-methionine had dissolved after 2 hours of shaking with 200 cc. of acetic acid and 22.5 cc. of acetic anhydride, and titration indicated complete acetylation. After distilling *in vacuo* and leaving the evaporation residue suspended overnight at  $-10^{\circ}$  in 80 cc. of toluene and 100 cc. of petroleum ether, the compound was obtained in 94 per cent yield. Equivalent weight (phenolphthalein) 191.1, 191.7; calculated 191.1. M.p. 113–113.5° (corrected); according to du Vigneaud and Meyer (22), 114–115° (corrected).

*N-Acetyl-l-hydroxyproline*—After 25 mm of *l*-hydroxyproline were shaken with 50 cc. of acetic acid and 3.4 cc. of acetic anhydride for 2 hours, the residue obtained on vacuum distillation was thoroughly digested, and left overnight in the cold, with ether. Yield 92 per cent, m.p. 131–132° (corrected),  $[\alpha]_D^{28.5} = -117.6^{\circ}$ ;  $[\alpha]_{H_2O}^{28} = -141.1^{\circ}$  (0.184 M in  $H_2O$ ), while Synge (18) obtained 133–134° and  $[\alpha]_D^{20} = -116.5^{\circ}$ . Equivalent weight 173.8; calculated 174.1.

*N-Acetyl-dl-tryptophane*—The solution resulting on shaking 10 mm of *l*-tryptophane with 20 cc. of acetic acid and 1.4 cc. of acetic anhydride for 2 hours was distilled *in vacuo*. The residue, after being dried over NaOH and  $H_2SO_4$ , was dissolved in 1 cc. of acetone. After 4 cc. of  $H_2O$  were added and the acetone allowed to evaporate at room temperature, the reaction product was obtained in 87 per cent yield. Equivalent weight 248.0; calculated 247.1. M.p. 203–204° (corrected); cf. du Vigneaud and Sealock (23) 205–206° (corrected). No optical rotation (1 per cent, neutralized solution in  $H_2O$ ).

*N-Formyl-dl-alanine*—To a solution of 25 mm of *dl*-alanine in 20 cc. of 98 per cent formic acid and 40 cc. of acetic acid 5 cc. of acetic anhydride were added. Evaporation over NaOH left a residue the weight of which

was 102 per cent of the calculated; and the equivalent weight found was 118.2, 118.4 (calculated 117.1), after recrystallization from ethyl acetate (38 cc. per gm., 65 per cent yield) 118.0, 118.3. In another experiment 15 cc. of 98 per cent formic acid and 5 cc. of acetic anhydride were mixed; immediate rise in temperature and incipient evolution of gas ensued. After 2 minutes the mixture was added to a solution of 25 mm of alanine in 5 cc. of formic acid, and diluted with 40 cc. of acetic acid. The results were weight of the evaporation residue 101 per cent, equivalent weight 117.5, 117.9; after recrystallization 117.7, 117.8. The different products melted between 144° and 146° (corrected); cf. Biilmann, Jensen, and Jensen (5) 147–148°.

*N-Formyl-dl-methionine*—A product of equivalent weight 180.4 (calculated 177.2) and melting point 99–100° (corrected) (cf. Windus and Marvel (24) 99–100°) was obtained in 95 per cent yield in the manner described for the second preparation of the alanine derivative.

*N,N'-Di-formyl-L-lysine*—After a solution of 6.25 mm of lysine dihydrochloride in 10 cc. of 98 per cent formic acid was shaken with a suspension of 12.5 mm of silver acetate in 14 cc. of acetic acid for 1½ hours, 20 cc. of a filtrate free of Cl<sup>-</sup> and Ag<sup>+</sup> were obtained. 2.4 cc. of acetic anhydride were added, and after 2 days standing the solution was left to evaporate in a desiccator (NaOH, oil). One recrystallization from an equal weight of water followed by two recrystallizations from twice the weight (of the dry material used) of water gave a product of constant melting point (132–133°) and equivalent weight (203.5, 202.8; calculated 202.1) in 51 per cent yield.  $[\alpha]_{\text{H}_2\text{O}}^{28} \approx +3.3^\circ$  and  $[\alpha]_{\text{D}}^{28} = +2.8^\circ$ , for the 0.34 M neutralized (NaOH) aqueous solution. A portion of this solution was mixed with 3 volumes of 20 per cent HCl, boiled for 1½ hours, and evaporated. The residue gave values for yield, acidity, and chloride which agreed satisfactorily with those calculated for the anticipated mixture of sodium chloride and lysine dihydrochloride. The values for the specific rotation of the resulting lysine,  $[\alpha]_{\text{D}}^{27} = +21.0^\circ$ ,  $[\alpha]_{\text{H}_2\text{O}}^{27} = +25.6^\circ$ , compared to those (+24.2°, +29.0°) obtained with a solution of pure L-lysine dihydrochloride<sup>4</sup> and sodium chloride of similar concentration, indicated that about 12 per cent racemization had occurred.

#### SUMMARY

An exploratory study has been made of the rates of formation of N-acetyl and N-formyl derivatives of amino acids by the action of acetic anhydride and formic acid-acetic anhydride in acetone solutions. It has been found

<sup>4</sup> The lysine used gave  $[\alpha]_{\text{D}}^{28} = +21.8^\circ$  (0.20 M dihydrochloride in H<sub>2</sub>O), in reasonable agreement with the determinations of Lutz and Jirgensons (13) if the fact that their  $[\alpha]$  values refer to the dihydrochloride as the unit is taken into account.

that the course of these reactions can be followed by titration with acetic acid, owing to the loss of basicity resulting from acylation of amino groups, and that the acetamido and formamido groups possess definite residual basic properties which are different and characteristic for each type. No major differences in the rates of the two types of reaction and in the reactivities of the different naturally occurring types of amino acids were encountered. However, certain characteristic tendencies in the behavior of the supernumerary amino groups of the basic amino acids were observed. It was found that conversion of the amino group into the ionic aminium form, by the addition of strong acid, greatly inhibits its reaction with acetic anhydride. Reaction products were isolated in a few cases.

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# THE INVESTIGATION OF AMINO ACID REACTIONS BY METHODS OF NON-AQUEOUS TITRIMETRY

## II DIFFERENTIAL ACETYLATION OF HYDROXY GROUPS, AND A METHOD FOR THE PREPARATION OF THE O-ACETYL DERIVATIVES OF HYDROXYAMINO ACIDS\*

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The present paper is intended as a contribution to the specific chemistry of the naturally occurring hydroxyamino acids. Certain established differences in the chemistry of the acyl derivatives of amino and hydroxy groups have so far not been utilized in the study of hydroxyamino acids, and in particular no simple method for the preparation of their O-acetyl derivatives has been developed. The long known catalytic effect of strong acids in the reaction of hydroxy groups with acetic anhydride has been the object of a quantitative study in acetic medium (Conant and Bramann (4)). On the other hand, it also has long been recognized (Pinnow (11)) that the acetylation of primary amines by acetic anhydride is greatly inhibited when they are present as salts of strong acids.<sup>1</sup>

The preceding paper (Kolb and Toennies (6)) contains evidence indicating that the acetylation of amino acids by acetic anhydride in acetic medium is greatly repressed by salt formation with perchloric acid, and an earlier study (Toennies and Elliott (20)) dealt with the accelerating effect in the same medium of perchloric acid on the acetylation of water by acetic anhydride. The present work shows that the catalyzed reaction of the —OH group of amino acids is very similar to that of water. The analytical determination of that reaction and its differentiation from the acetylation of amino groups is accomplished as follows: If an acetic solution of an

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<sup>1</sup> Whenever acidity has been found beneficial in the acetylation of amines (Vorlander and Mumme (23), Reverdin and Crépieux (12), Smith and Orton (13)), the latter have been compounds of very weak basicity in which the extent of salt formation may be assumed to be negligible. That in those cases the accelerating effect of strong acids should prevail is logical if the latter involves an activation of the acetic anhydride rather than of the substrate to be acetylated, a view which is borne out by available evidence on the formation of active compounds between acetic anhydride and strong acids (Stillich (15), Murray and Kenyon (9)).



amino acid, acidified by perchloric acid and containing acetic anhydride, is added to an amount of anthranilic acid (or other suitable amine) which is more than equimolar to the sum of the free perchloric acid and the available acetic anhydride present, then the free amino groups will react with the available acetic anhydride forming N-acetyl derivatives. To the extent that the latter are devoid of basic properties titration with perchloric acid, after completion of the reaction between acetic anhydride and amino groups, will determine the remaining amino groups, and, if their initial amount (including those of anthranilic acid) is known, the amount which has reacted with, *i.e.* which is equimolar with, the available acetic anhydride.<sup>2</sup> The difference between this and the initially used acetic anhydride will be a measure of the acetic anhydride consumed by reaction with  $-\text{OH}$  groups while the solution was in the acid state. However, a kinetic study of the acid-catalyzed reaction of hydroxy groups with acetic anhydride will be possible only if in the basic state the reaction of  $-\text{OH}$  with acetic anhydride is negligibly slow compared with that of  $-\text{NH}_2$ . This was found to be the case. On the other hand, the acetylation of  $-\text{NH}_2$  groups while the solution is in the acid state was not found to be entirely negligible. The extent of this reaction is determined by a separate titration with acetic sodium acetate, which will reveal conversion of basic  $-\text{NH}_2$  groups to non-basic acetamino groups, but the determination of the O-acetylation will not be affected by it. Problems of analytical detail are discussed in the experimental part.

The finding that the speed of O-acetylation increases with increasing concentration of perchloric acid while the speed of N-acetylation decreases showed the way for a practical method for the preparation of O-acetyl derivatives of hydroxyamino acids. Acetylation with acetic anhydride in the presence of an excess of perchloric acid and decomposition of the remaining acetic anhydride by water is followed by neutralization with amylamine, of which the perchlorate is soluble in practically all organic solvents. The O-acetyl derivatives of serine, threonine, tyrosine, and hydroxyproline are in their isoelectric form only moderately soluble in acetic acid, and by lowering their solubility through addition of ether or similar liquids they can be precipitated in yields of 80 to 90 per cent. By comparison the solubility of the N-acetylamino acids in the organic media used is high; so that any that may be present as by-products are eliminated in the precipitation process.

Heretofore O-acetylamino acids have apparently not been prepared. Suggestions as to their probable properties and as to possible alternative modes for their formation could be gleaned from some available data per-

<sup>2</sup> To our knowledge this scheme for the determination of acetic anhydride was first utilized by Kilpi (5).

taining to the analogous O-benzoyl compounds. Sørensen and Andersen (14), later confirmed by Synge (16), showed that in O-, N-diacyl derivatives alkaline hydrolysis causes easy cleavage of the O-acyl group without affecting the N-acyl bond. That conversely under conditions of acid hydrolysis the N-acyl linkage is split more easily than the O-acyl linkage was shown by Synge (17). The opposite relation to acidity of the two types of acyl derivatives extends even further. Just as N-acylation is inhibited by acid conditions, O-acylation is inhibited by alkaline conditions. This is indicated in the study of Sørensen and Andersen (14) who found that in treating  $\alpha$ -amino- $\delta$ -hydroxyvaleric acid with benzoyl chloride in a strongly (0.5 N) alkaline medium only the  $-\text{NH}_2$  groups are benzoylated, whereas the  $-\text{OH}$  group also reacts when the procedure is carried out in a nearly neutral medium. Similarly Bergmann and Zervas (3) and du Vigneaud and Meyer (22) have shown that treatment of tyrosine with acetic anhydride leads to the formation of the N-acetyl derivative when the aqueous medium is strongly alkaline, while under less alkaline conditions the O-, N-diacetyl compound results. Finally, reference must be made to the studies of Bergmann *et al.* (1, 2) on rearrangements in acyl derivatives of hydroxyamino acids; again it emerges that *acidity favors O-acylation, while alkalinity favors N-acylation*.

Some of the relations outlined have been utilized for the preparation of amino acid O-acyl derivatives by Bergmann *et al.* (1, 2) and by Synge (17). The former obtained the O-benzoyl derivatives of  $\gamma$ -amino- $\beta$ -hydroxybutyric acid and serine from the corresponding N-benzoyl compounds by anhydration to the 2-phenyloxazoline ring and its reopening under acid conditions. The latter obtained O-benzoylhydroxyproline and O-benzoylserine in small yields by acid hydrolysis of the N-acetyl-O-benzoyl derivatives. Our approach to the corresponding O-acetyl derivatives is more direct as well as productive of better yields than these methods. Moreover, its applicability is not limited to compounds in which the hydroxy and the amino groups are attached to adjoining carbon atoms, as is the oxazoline method. Suitable modifications of our procedure, involving the use of benzoic or other anhydrides and the corresponding or inert solvents, may permit its extension to the preparation of other O-acyl derivatives. The preparation of O-, N-diacetyl derivatives, from the O-acetyl derivatives by the technique outlined in the preceding paper (Kolb and Toennies (6)), should offer no difficulties.

#### EXPERIMENTAL

*Analytical Reagents*—The acetic anhydride used in the present work was analyzed by several methods, with the following results. Our customary methylate method (Toennies and Elliott (20)) gave 9.29 mm of acetic

anhydride per gm., the iodometric dichloroaniline method of Orton and Bradfield (10) gave 9.30 mm, and the anthranilic acid method (detailed below) gave 9.32 mm; the average value is  $9.30 \pm 0.01$ .

A standard acetic perchloric acid solution<sup>3</sup> was prepared by adding to a weighed amount of concentrated aqueous perchloric acid, dissolved in acetic acid (U.S.P.), an amount of acetic anhydride equimolar to the water accompanying the perchloric acid.<sup>4</sup>

When anthranilic acid as purchased was not of satisfactory purity (white crystals, titration value against acetic perchloric acid at least 99.5 per cent of the theoretical), it was purified by several recrystallizations from water and from alcohol. Fresh acetic solutions of anthranilic acid show a characteristic bluish fluorescence, and on standing develop a yellow color. This discoloration process, very rapid in direct sunlight, is practically eliminated in the dark. Besides, a very slow spontaneous acetylation occurs, but its extent is negligible for at least 20 hours in an approximately 0.5 M solution in anhydrous acetic acid.

*Determination of Acetic Anhydride with Anthranilic Acid*—In contrast to the behavior of acetyl- and formylalanine (Kolb and Toennies (6)) the basic effect of acetyl-anthranilic acid in the perchloric acid titration is

<sup>3</sup> In defining normalities of acetic solutions in the present work effective normalities have been used throughout unless otherwise stated; i.e., (a) the normality has been corrected for the temperature of the titration by using the expansion coefficient 0.107 per cent per degree, and (b) instead of deducting the experimental solvent blank correction (cf. Toennies and Callan (18)) for the volume titrated, this correction has been incorporated in the normality value. For instance, in reference to the perchloric acid solution mentioned it was found that 10 cc. of the acetic acid used in its preparation have a blank value of 0.07 cc. of 0.1 N  $\text{HClO}_4$ . Only a single end-point, viz. the point "at which emerald-green has just turned yellow-green," was used in the present work. The selection of a suitable end-point shade is a matter of the individual preference of the operator. In the standardization of the perchloric acid solution against glycine (Toennies and Callan (18)), instead of making *pro rata* deductions of 0.07 cc. per 10 cc. consumed, the normality was calculated without making deductions. A value (at 25°) of 0.0996 N ( $\pm 0.1$  per cent) was obtained, and this value was used in practice as the titrating normality. However, by making the proper blank correction a value of 0.1003 N would result for the true normality, and this value compares well with the value of 0.1004 N obtained by calculation from the amount of aqueous perchloric acid used.

<sup>4</sup> In making up this solution the following technique, convenient for obtaining desired amounts of concentrated reagent solutions, was used. A 25 cc. glass-stoppered volumetric flask, freshly cleaned with bichromate-sulfuric acid and dried, was filled with some perchloric acid, thoroughly rinsed, emptied, drained for 60 seconds, and weighed. The desired amount of perchloric acid was then weighed into the flask, and by emptying it under the same conditions of draining as before the weighed amount is obtained within close limits.

nearly negligible: 0.6 mm of acetylanthranilic acid<sup>5</sup> titrated in the presence of 10, 20, or 30 cc. of acetic acid consumed 0.07, 0.13, and 0.19 cc. of 0.1 N HClO<sub>4</sub>, while the corresponding solvent blanks were 0.06, 0.12, and 0.18 cc.

In order to establish the rate of reaction between acetic anhydride and anthranilic acid under the conditions encountered in the intended kinetic experiments, acetic acid solutions of the two compounds were combined in such amounts as to produce a solution of 0.093 M acetic anhydride and 0.139 M anthranilic acid. The mixture was kept in a bath at  $25.30^{\circ} \pm 0.05^{\circ}$ , and, at intervals, 5 cc. portions were withdrawn and titrated with acetic perchloric acid, with the following results.

Time, min.	5	15	30	60	120	180	1400
HClO <sub>4</sub> , m eq.	0.427	0.309	0.251	0.235	0.233	0.232	0.232

These results are in harmony with a bimolecular velocity constant (moles per liter per minute) of 0.156 ( $\pm 4$  per cent), and it may be concluded from them that as long as the temperature and the ratio as well as the absolute values of the concentrations are not lower than those used here a period of 3 hours is amply sufficient for complete reaction.

In a practical method for the determination of acetic anhydride account must be taken of the water ordinarily present in the acetic acid used as the medium. Its concentration is, according to our experience, approximately 0.1 M. Its effect was examined as follows: Into seven 50 cc. glass-stoppered Erlenmeyer flasks, identical 4 cc. portions of an 0.5 M anthranilic acid solution were pipetted. To two of these, 10 cc. portions of a freshly prepared approximately 0.13 M solution of acetic anhydride (exactly weighed) were added. This occurred approximately 5 minutes after the concentrated acetic anhydride had been diluted with acetic acid. 10 minutes later another two 10 cc. portions of the same solution were added to anthranilic acid flasks. All flasks (three of them as controls) were titrated after 3 hours standing. The experiment was repeated after the water content of the acetic acid used in dissolving the acetic anhydride was increased by 0.1 mole per liter. In other experiments some organic hydroxy compounds in 0.1 M concentration were added, instead of water, to the acetic acid. The compounds used were ethanol, menthol, resorcinol,

<sup>5</sup> Acetylanthranilic acid was simply obtained by dissolving 100 mm of anthranilic acid in 150 cc. of acetic acid and adding 100 mm of acetic anhydride. The acetyl derivative crystallized; it was filtered after several hours and dried in the air in a warm place. Yield about 60 per cent. Equivalent weight (NaOH, phenolphthalein) found 177.7, calculated 179.1.

and hydroquinone. In all cases (addition of water and of hydroxy compounds, time of interaction with acetic anhydride previous to combination with anthranilic acid solution either 5 or 15 minutes) the resulting value for acetic anhydride was within  $\pm 0.2$  per cent of that obtained with unadulterated acetic acid. One may conclude, therefore, that under the experimental conditions organic hydroxy groups are not likely to interfere with the determination of acetic anhydride, and that, likewise, moderate amounts of water can be disregarded.

On the other hand, in the kinetic experiments described below the solution in which the amount of free acetic anhydride is to be determined contains free perchloric acid which in the concentrations present (0.02 M) renders the interaction of water with acetic anhydride extremely rapid (cf. Toennies and Elliott (19)). Rather than to determine whether the acid can be a cause of errors by producing a certain amount of reaction of anhydride with the water present in the anthranilic acid solution, during the process of adding the acidified anhydride solution to the latter, it was considered simpler to employ dehydrated acetic acid for the anthranilic acid solutions.

*Preparation and Analysis of Dehydrated Acetic Acid*—To 10 liters of acetic acid 200 cc. of 10.04 M acetic anhydride and 100 cc. of 0.100 N acetic perchloric acid were added. After 2 days the resulting solution was analyzed for free acetic anhydride in the manner described, on 10 cc. portions, with 4 cc. portions of 0.5 M acetic anthranilic acid. The difference in titration between tests and anthranilic acid blanks (three of each) was  $9.42 \pm 0.02$  cc. of 0.100 N  $\text{HClO}_4$ . From this must be deducted 0.10 cc. for the perchloric acid present (0.001 M) in the solution that is being analyzed, so that an acetic anhydride concentration of 0.0932 mole per liter results for the dehydrated solution, or a total amount, assuming additive volumes, of 960 mm. Since 2008 mm of acetic anhydride were added, 1048 mm of water have reacted; *i. e.*, the original water content was 0.104 mole per liter. The analysis of the dehydrated solution was repeated, after 2 months standing, with essentially the same results.

The blank titration value of the ordinary acetic acid is about 0.15 cc. of 0.1 N  $\text{HClO}_4$  per 25 cc.; that of the dehydrated solution corresponds to only 0.05 cc. per 25 cc., since back titration of the solution, which according to the perchloric acid present should take 0.25 cc. of 0.1 N acetic sodium acetate, requires only 0.20 cc.

*Preparation and Analysis of Anhydrous Perchloric Acid Solutions*—In measurements of the acid-catalyzed acetylation of hydroxy groups water is, of course, a source of error. For this reason perchloric acid solutions as nearly anhydrous as possible were prepared for use in the reaction mixtures to be investigated. Three different concentrations of perchloric acid, 0.10 M, 0.125 M, and 0.50 M, were prepared in this connection.

To 1000 cc. of the dehydrated acetic acid (0.001 M  $\text{HClO}_4$ , 0.0932 M  $(\text{CH}_3\text{CO})_2\text{O}$ ) 14.57 gm. of  $\text{HClO}_4$  (68.42 per cent) and 16.53 gm. of  $(\text{CH}_3\text{CO})_2\text{O}$  (95.1 per cent) were added. According to calculation the resulting solution should be 0.0978 M in  $\text{HClO}_4$  and should have a residual water content of 0.009 mole per liter. Standardization by titration of dry samples of anthranilic acid and against a standardized sodium acetate solution gave the value of  $0.0976 \pm 0.002$  N for  $\text{HClO}_4$ , and instead of the estimated residual water content a slight excess of acetic anhydride was found,<sup>6</sup> by using the following analytical procedure.

A 6 cc. portion of a 0.5 M anthranilic acid solution was titrated with the perchloric acid solution to be analyzed, 30.80 cc. being required. By running this amount into another flask, adding a 6 cc. portion of anthranilic acid to it, and completing the titration, the total  $\text{HClO}_4$  required was (two experiments) 30.87, 30.90 cc. The higher value is presumably due to the fact that in the first experiment (direct titration) some of the anthranilic acid (base) is "neutralized" by being acetylated by acetic anhydride present in the  $\text{HClO}_4$  solution. If the two solutions are combined in the reverse order, the prevailing acid reaction will prevent the acetylation. Now three additional 6 cc. portions of the anthranilic acid solution were pipetted out and 10.00 cc. of perchloric acid were added to each. After 4 hours one was titrated, resulting in a total consumption of 30.52 cc. of  $\text{HClO}_4$  solution. When this amount was added rapidly to the other two flasks and then the titration completed, 30.52 and 30.54 cc. were used. If the difference between the total anthranilic acid titrated above ( $30.89 \pm 0.02$  cc. of  $\text{HClO}_4$ ) and that titrated after the solution had been in contact with 10 cc. of the  $\text{HClO}_4$  solution for 4 hours is assumed to be the result of acetylation by acetic anhydride present in the 10 cc., an acetic anhydride content of the perchloric acid solution of 0.0035 mole per liter is indicated.

By similar procedures 1 liter of a 0.1252 N  $\text{HClO}_4$  solution was prepared and analyzed. In this case the balance between acetic anhydride and water should, according to calculation, have resulted in a residual water content of 0.0005 mole per liter; analysis, however, indicated the presence of 0.0034 M acetic anhydride.<sup>6</sup>

Finally 1 liter of a 0.5 M anhydrous  $\text{HClO}_4$  solution was prepared, likewise by first dissolving the concentrated aqueous perchloric acid in the dehydrated acetic acid and then adding the requisite amount of acetic anhydride. As in this case the heat of reaction was considerable and since the resulting solution is notably more viscous than the less concentrated

<sup>6</sup> Other, unpublished, experiments support the suggestion conveyed by these data that the reaction  $(\text{CH}_3\text{CO})_2\text{O} + \text{H}_2\text{O} \rightarrow 2\text{CH}_3\text{COOH}$  may not be entirely irreversible.

ones, it was deemed neither safe nor expedient to operate with anhydrous aceteous perchloric acid solutions more concentrated than 0.5 M.

*Inhibition of N-Acetylation by Acidity*—In the preceding paper (Kolb and Toennies (6)) an experiment has been presented which shows that the N-acetylation of an amino acid (alanine) is extremely slow in the presence of an excess of perchloric acid. In the present work additional data on this point, related to the conditions employed in the study of the hydroxy acetylations, have been obtained. Equal amounts (216.8 mg) of *dl*-alanine were dissolved (a) in 25.02 cc of an aceteous perchloric acid solution (0.0973 N  $\text{HClO}_4$ , 0.0032 M  $(\text{CH}_3\text{CO})_2\text{O}$ ), i.e. an equivalent amount, (b) in 25 cc. of a solution obtained by diluting 0.5 cc of an anhydrous

TABLE I  
Effect of Excess  $\text{HClO}_4$  on N-Acetylation of Alanine

*dl*-Alanine perchlorate 0.087 M, acetic anhydride 0.130 M, medium acetic acid, temperature  $25.2^\circ \pm 0.2^\circ$ . The increase in sodium acetate titration value is taken as a measure of the conversion of basic amino groups into non basic acetamino groups. The extent of this reaction is calculated in per cent of the alanine present.

Time	0.003 M excess $\text{HClO}_4$		0.004 M excess $\text{HClO}_4$		0.015 M excess $\text{HClO}_4$		0.036 M excess $\text{HClO}_4$		0.072 M excess $\text{HClO}_4$	
	0.1 N $\text{CH}_3\text{COONa}$ solution	Acetylation	0.1 N $\text{CH}_3\text{COONa}$ solution	Acetylation	0.1 N $\text{CH}_3\text{COONa}$ solution	Acetylation	0.1 N $\text{CH}_3\text{COONa}$ solution	Acetylation	0.1 N $\text{CH}_3\text{COONa}$ solution	Acetylation
hrs	cc	per cent	cc	per cent	cc	per cent	cc	per cent	cc	per cent
0.15					0.79		1.90		3.94	
0.5			0.26							
1.0	0.08	1.8	0.25	-0.2	0.80	0.2	1.90		3.95	0.2
11	0.26	6.0	0.30	0.9	0.82	0.7	1.93	0.7	3.97	0.7
22	0.32	7.3	0.34	1.8	0.82	0.7	1.92	0.5	3.97	0.7

0.5 M  $\text{HClO}_4$  solution to 50 cc with the solution mentioned under (a), (c) similarly except that the amount of 0.5 M  $\text{HClO}_4$  used was 2 cc, (d) similarly except with 5 cc. of 0.5 M  $\text{HClO}_4$ , and (e) similarly except with 10 cc. of 0.5 M  $\text{HClO}_4$ . Each solution was combined with 2.5 cc of a 1.42 M solution of acetic anhydride, and at intervals 18.0 per cent aliquots (5 cc, determined by weight) were withdrawn and titrated with a 0.1006 N aceteous sodium acetate solution. The experiments were conducted at  $25.4^\circ \pm 0.2^\circ$ . The results are summarized in Table I. It appears that the rate of N-acetylation decreases with increasing acidity, but that practically no further suppression is achieved by an excess of perchloric acid greater than 20 per cent.

*Reaction of Hydroxyamino Acids with Acetic Anhydride in Presence of Perchloric Acid*—The compounds used for the kinetic experiments were

dried, pulverized, passed through a 100 mesh sieve, and dried again, for 1 hour periods, at  $100^{\circ}$  until of constant weight. Samples were then analyzed by perchloric acid titration, with the following results: hydroxyproline, equivalent weight found 131.1 (theoretical 131.1), serine 105.3 (theoretical 105.1), threonine 119.0 (theoretical 119.1), tyrosine 180.9 (theoretical 181.1).

The reaction of the four available hydroxyamino acids with acetic anhydride in acetic acid was studied under two conditions of acidity, Series A in the presence of an equimolar amount of  $\text{HClO}_4$ , and Series B in the presence of an excess of approximately 28 per cent, while in both cases the amino acid concentration was about 0.09 M, and about 1.4 molecules of acetic anhydride were available for each molecule of amino acid. Furthermore, in one case (Series C, tyrosine) the course of the reaction was followed at substantially higher concentrations, *viz.* approximately 0.4 M amino acid, in the presence of 1.2 molecules of perchloric acid and 1.4 molecules of acetic anhydride per molecule of amino acid, in order to establish the absence of complications when the concentrations are multiplied. This experiment was carried out primarily with a view toward the isolation of the expected O-acetyl derivatives.

The procedures were as follows: In Series A, anhydrous approximately 1.4 M acetic anhydride solution was prepared by diluting 15 cc. of 95.1 per cent acetic anhydride, together with 10 cc. of 0.1 N acetic  $\text{HClO}_4$ , to 100 cc. with ordinary acetic acid. After 24 hours were allowed for the acid-catalyzed acetylation of the available water, the acetic anhydride content of the solution was determined by the anthranilic acid (4 cc. of 0.5 M) method on weighed 0.9 cc. portions. Then exactly 5.00 mm of the respective amino acid were weighed out into a 100 cc. glass-stoppered flask, and a volume of an anhydrous, approximately 0.1 N (acidity value and that of the small excess of acetic anhydride present accurately determined)  $\text{HClO}_4$  solution was added which corresponds to 4.95 milliequivalents. After the amino acid had dissolved, the flask was placed in the bath at  $25.4^{\circ} \pm 0.1^{\circ}$  and, after at least 10 minutes were allowed for temperature adjustment, 5 cc. of the above acetic anhydride solution were added, with thorough agitation. Since the acetic anhydride solution is 0.01 N in  $\text{HClO}_4$ , the total perchloric acid is now 5.00 mm; *i.e.*, equivalent to the amino acid. At selected intervals, counting from the time of addition of acetic anhydride, 5 cc. portions of the reaction mixture were withdrawn and immediately titrated back with 0.1 N sodium acetate solution. Other 5 cc. portions were directly pipetted into 2 cc. portions of a 0.5 M anthranilic acid solution and, after 3 hours, titrated with acetic  $\text{HClO}_4$ , together with anthranilic acid blanks. The amount of perchloric acid solution used in the reaction mixture had been weighed; by determining the weight of 5



cc. portions of the acetic anhydride solution used and of some of the 5 cc. portions withdrawn from the reaction mixture for analysis, all necessary data were obtained for calculating the composition of the reaction mixture and the weight fraction of the analytical portions, so that the results could be calculated in terms of the total reaction mixture without the complicating necessity of making the reaction mixtures to a definite volume and other difficulties. In the reactions of Series B the procedure was similar except that the perchloric acid used in the reaction mixture was about 0.125 N. In the single experiment which was carried out at a substantially higher concentration, Series C, 4.96 mm of tyrosine were dissolved in 12 cc. of an anhydrous 0.503 M perchloric acid solution. After the mixture was weighed and had reached the temperature of the bath, concentrated acetic anhydride corresponding to 7.20 mm was added, and 1 cc. portions of the reaction mixture were used for the determinations.

The results of these experiments are presented in Table II. It appears that the rate of N-acetylation, both in the absence of free perchloric acid and in its presence, is lowest in the case of tyrosine and highest in the case of hydroxyproline. Furthermore, the velocity of this reaction is, in confirmation of what had already been demonstrated for alanine (Table I), in all cases decreased by the presence of free perchloric acid. The velocity of O-acetylation, on the other hand, is always increased by the addition of acid. Among the different compounds the reaction of the hydroxy groups seems likewise to be slowest in the case of tyrosine and fastest in the case of hydroxyproline, but the relative position of the other two representatives, serine and threonine, may not be the same here as in the reactivity of the amino groups. In appraising the order in which the reactivity of the four compounds appears in experimental Series A, one must consider the possibility that the relative position may in part be the result of small variations, beyond the experimental precision, in the acid-base ratio, since the balance in this series is one of "neutrality" ( $\text{NH}_2$  equal to  $\text{HClO}_4$ ); *i.e.*, possibly a range highly sensitive to small variations. However, the fact that the relative order of the four compounds is the same in the "neutral" series as in that containing an excess of acid speaks against the importance of this source of error.

The small deviations from the theoretical value of 100 per cent which are in evidence in the O-acetylation data of Table II are thought to be the combined result of imperfect purity of the compounds used and of analytical errors. The latter are especially prominent in the more concentrated system of Series C where increased viscosity of the solution and small size of samples are distinct sources of error.

*Preparation of Acetoxyamino Acids*—The general procedure developed for purposes of isolation is as follows: By appropriate dilution of the con-

centrated aqueous perchloric acid with acetic acid an acetous solution 0.60 M in  $\text{HClO}_4$  and about 1.7 M in  $\text{H}_2\text{O}$  is prepared. 100 cc. of this solution are added to 50 mm of the pulverized hydroxyamino acid. After solu-

TABLE II

*Rates of Acetylation of Amino and Hydroxy Groups of Hydroxyamino Acids*

The experimental conditions described in the text were used. The values are given in per cent.

Time*	Tyrosine		Serine		Threonine		Hydroxyproline	
	Groups acetylated							
	—NH <sub>2</sub>	—OH	—NH <sub>2</sub>	—OH	—NH <sub>2</sub>	—OH	—NH <sub>2</sub>	—OH
Series A. 0.038 M amino acid, 0.038 M perchloric acid, 0.123 M acetic anhydride								
hrs.								
1	3.0	70.2	3.0	94.6	5.1	85.4	8.6	96.4
3		93.6		97.5		99.0		98.2
6.5	6.2	97.4	8.7	98.3	12.5	101.1	22.4	99.4
14.5	9.8	99.0	12.8	98.5	18.4	100.8	29.0	100.0
23.5	13.6	98.8	16.2	98.7	21.9	101.2	35.4	100.2
Series B. 0.088 M amino acid, 0.113 M perchloric acid, 0.123 M acetic anhydride								
0.25		99.2		100.2		100.0		98.8
1	-0.4	100.6	-1.2	100.6	-0.2	100.0	0.0	99.0
3		101.0		100.8		101.2		99.6
6.5	-0.2	101.6	0.0	102.2	1.6	101.6	4.2	99.8
14.5	0.6	102.2	0.8	102.4	3.6	101.4	8.8	99.6
Series C. 0.368 M amino acid, 0.447 M perchloric acid, 0.530 M acetic anhydride								
0.03	0.0							
0.23		103						
0.25	0.2							
0.5		103						
0.75	0.0							
1.0		104						
1.25	0.2							
1.5		103						
1.75	0.2							
2.0		103						

\* In Series A and B the determination of —NH<sub>2</sub> acetylation (titration with sodium acetate) was performed approximately 5 minutes after the stated times.

tion is complete, acetic anhydride is added. Its amount corresponds, first, equimolarly to the total water present, and, in addition, to a 40 per cent excess over that required for the O-acetylation. Because under the present

conditions the heat of acetylation is considerable, it is advisable to run the concentrated acetic anhydride slowly into the solution, which is cooled by gentle swirling in an ice bath. The combined solution is kept in a glass-stoppered flask for about 1 hour at room temperature, in order to insure completion of the O-acetylation. Thereupon 2 cc. of water (110 mm) are added which under the prevailing conditions of acid catalysis will eliminate all remaining acetic anhydride. If the latter were not thus removed, it would react with amino groups when the solution is made basic in the next step, and thus could interfere with yield and purity of the reaction product. After 1 hour is allowed for the hydration of the residual acetic anhydride, 80 mm of commercial amylamine (Sharples Solvents Corporation; approximately 8.0 M) are added to the solution with cooling. In some cases the reaction product will begin to precipitate on standing; it is obtained promptly and in good yield by the addition of a suitable precipitating liquid, such as alcohol, acetone, ether, chloroform, etc. The exact choice for each compound under consideration is best determined by experiment, and the solvent combinations stated below have been thus found. After at least one night in the refrigerator the precipitate is filtered and washed with ether until the acidity of the washings does not decrease further.<sup>7</sup> The product is cautiously (some O-acetyl derivatives show signs of decomposition at 100°) dried to constant weight and analyzed. Data about the individual compounds are summarized in Table III. The evidence for the identity of the isolated products consists of (a) the data of Table II which show that the amino acids used consume quantitatively 1 molecule of acetic anhydride without involvement of the amino group, (b) the equivalent weights obtained by perchloric acid and methylate titrations which are in harmony with an increase of the molecular weight of the parent compound by the value corresponding to  $\text{CH}_2\text{CO}$ , and which remain essentially unchanged after a fractionating recrystallization, (c) the essentially identical equivalent weights of the compounds as bases (by  $\text{HClO}_4$ ) and as acids (by methylate), which are indicative of the amphoteric nature of a monoaminomonocarboxylic acid, and (d) a polarimetric analysis in the case of the tyrosine and hydroxyproline derivatives (see below).

The observed deviations in the acid values do not seem to be due entirely to lack of purity but may be related to the basic properties of the compounds (*cf.* Toennies and Callan (18)). All perchloric acid titrations could be carried out without the use of formic acid, although the recrystallized specimens, because of their more coarsely crystalline nature, required considerable intermittent heating in order to complete the titrations. As stated in Table III, the tyrosine derivative showed abnormal behavior

<sup>7</sup> It is convenient to determine this by titrating portions of the ether washings with sodium methylate and thymol blue indicator (Lavine and Toennies (7)).

TABLE III  
Preparation of Acetoxyamino Acids

For other details, see the text.

Starting material		O-Acetyl derivative, isolation					Recrystallization of derivative				
Compound (mol. wt.)	Equivalent weight found (HClO <sub>4</sub> )	Solvent combination used for pptn.	Yield	Theoretical mol. wt.	Equivalent weight found* by titration with		Solvent (50 per cent alcohol) used	Yield	Equivalent weight found* by titration with		Decomposition point
					HClO <sub>4</sub>	CH <sub>3</sub> ONa†			HClO <sub>4</sub>	CH <sub>3</sub> ONa†	
<i>l</i> -Hydroxyproline (131.1)	130.7	200 cc. butyl ether + 2000 cc. ethyl ether‡	83	173.1	176.5	174.0	0.37	23	176.5	173.9	179-181
<i>dl</i> -Serine (105.1)	106.3	1200 cc. ethyl ether	95	147.1	149.5	146.9	0.70	89	147.3	149.7	143-144§
<i>dl</i> -Threonine (119.1)	117.6	50 cc. butyl ether + 1000 cc. ethyl ether	84	161.1	168.3	162.6	2.7	65	164.8	162.5	146-149§
<i>l</i> -Tyrosine (181.1)	184.7	250 cc. acetone + 750 cc. ethyl ether	91	223.1	223.7		2.6	68	224.1		213-214

\* The figures given are the averages of determinations carried out at least in duplicate.

† 0.1 N CH<sub>3</sub>ONa, standardized against benzoic acid; indicator, thymol blue; end-point, from yellow to blue; blank correction to be deducted obtained by titrating to the same color after addition to the titrated solution of methanol and indicator in amounts equal to those already present.

‡ The precipitate was oily at first, but after 5 days in the cold, with repeated agitation, all had solidified. In another experiment on a smaller scale (5 mm) a well crystallized product was obtained at once by the use of 2½ volumes of methyl cello-solve (CH<sub>3</sub>OCH<sub>2</sub>CH<sub>2</sub>OH) and 20 volumes of ether.

§ With gas evolution.

|| No definite end-point could be obtained; apparently the acetyl group is slowly split off by the methylate.

in the methylate titration. Titration of the new compounds by the formol method was attempted, but no definite end-points could be obtained because of hydrolysis of the compounds on addition of alkali.

Comparison of the decomposition points (last column, Table III) with

conditions the heat of acetylation is considerable, it is advisable to run the concentrated acetic anhydride slowly into the solution, which is cooled by gentle swirling in an ice bath. The combined solution is kept in a glass-stoppered flask for about 1 hour at room temperature, in order to insure completion of the O-acetylation. Thereupon 2 cc. of water (110 mm) are added which under the prevailing conditions of acid catalysis will eliminate all remaining acetic anhydride. If the latter were not thus removed, it would react with amino groups when the solution is made basic in the next step, and thus could interfere with yield and purity of the reaction product. After 1 hour is allowed for the hydration of the residual acetic anhydride, 80 mm of commercial amylamine (Sharples Solvents Corporation; approximately 8.0 M) are added to the solution with cooling. In some cases the reaction product will begin to precipitate on standing; it is obtained promptly and in good yield by the addition of a suitable precipitating liquid, such as alcohol, acetone, ether, chloroform, etc. The exact choice for each compound under consideration is best determined by experiment, and the solvent combinations stated below have been thus found. After at least one night in the refrigerator the precipitate is filtered and washed with ether until the acidity of the washings does not decrease further.<sup>7</sup> The product is cautiously (some O-acetyl derivatives show signs of decomposition at 100°) dried to constant weight and analyzed. Data about the individual compounds are summarized in Table III. The evidence for the identity of the isolated products consists of (a) the data of Table II which show that the amino acids used consume quantitatively 1 molecule of acetic anhydride without involvement of the amino group, (b) the equivalent weights obtained by perchloric acid and methylate titrations which are in harmony with an increase of the molecular weight of the parent compound by the value corresponding to  $\text{CH}_3\text{CO}$ , and which remain essentially unchanged after a fractionating recrystallization, (c) the essentially identical equivalent weights of the compounds as bases (by  $\text{HClO}_4$ ) and as acids (by methylate), which are indicative of the amphoteric nature of a monoaminomonocarboxylic acid, and (d) a polarimetric analysis in the case of the tyrosine and hydroxyproline derivatives (see below).

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<sup>7</sup> It is convenient to determine this by titrating portions of the ether washings with sodium methylate and thymol blue indicator (Lavine and Toennies (7)).

the presence of excess perchloric acid, the hydroxyamino acids may be quantitatively acetylated solely on the hydroxyl group. A method for the preparation of the new compounds O-acetylhydroxyproline, O-acetylserine, O-acetylthreonine, and O-acetyltyrosine, based on the findings outlined, is presented. The method is useful for the preparation of other related compounds.

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# THE INVESTIGATION OF AMINO ACID REACTIONS BY METHODS OF NON-AQUEOUS TITRIMETRY

## III. THE DETERMINATION OF HYDROXY (AND ANALOGOUS) GROUPS IN AMINO ACIDS\*

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In Paper II of this series (Sakami and Toennies (3)) it was shown that in an acid acetous medium the hydroxy groups of amino acids react quantitatively with acetic anhydride and that the extent of this reaction can be determined by measuring the resulting decrease in acetic anhydride available for reaction with amino groups under basic conditions, the latter reaction being accompanied by loss of titratability of the amino groups. The change from an acid to a basic condition is accomplished by the addition of anthranilic acid (a base in the acetous acid-base scheme), which likewise supplies an excess of amino groups. In the present paper we describe a practical procedure for, and the results of, the analytical application of these principles. By the method evolved available hydroxy groups can be determined with a precision of approximately  $\pm 0.003$  milliequivalent when applied to about 0.3 mM of dry amino acids.

### Outline

About 0.3 mM of amino acid, exactly weighed, is dissolved in 4 cc. of an acetous solution (acetylation mixture) which is 0.2 to 0.25 M in both perchloric acid and acetic anhydride. After 2 hours are allowed for the reaction of the acetic anhydride in the acid medium, 3 cc. of the reaction mixture are pipetted into 4 cc. of an 0.4 to 0.45 M acetous anthranilic acid solution. The exact amount of each of these portions is determined by weighing. After 3 hours are allowed for the reaction of the acetic anhydride in the basic medium, the solution is titrated with 0.1 N acetous  $\text{HClO}_4$  in the presence of crystal-violet indicator. A blank value is obtained by running a parallel determination in which only the amino acid is omitted. If the amounts of reagents in both determinations were exactly identical, and the partial volume of the amino acid were negligible, the first titration value should be larger than the second by an amount corresponding to the amino groups of the amino acid and the amino groups eliminated from reaction with acetic anhydride (in the basic stage) by

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reaction of the latter (in the acid stage) with hydroxy, or analogous, groups. The amount of these groups (corresponding to three-fourths of the substance used) would then be obtained by deducting from the titration difference the  $-\text{NH}_2$  value of the amount of amino acid involved, as derived from a separate  $\text{HClO}_4$  titration. In practice the  $-\text{OH}$  value thus obtained is subject to considerable error, chiefly because of differences in the actual amounts obtained in the corresponding pipetted portions of the two determinations. However, knowledge of the actual weights of the portions and of the net acid or base value of the solutions employed makes it possible to correct for these differences.

*Reagents*—Acetuous 0.1 N  $\text{HClO}_4$  and the indicator solutions were prepared and used as described by Toennies and Callan (4), except that diphenylguanidine has now been substituted for glycine as the primary standard. Its advantages are a large equivalent weight and easy solubility, and the fact that it can be used equally well for the standardization of aqueous (Young (7)) and acetuous solutions. By reference to both systems, glycine (Amino Acid Manufactures, analytically pure, Lot 8) and borax (Hurley (1)) being used as the respective primary standards, Eastman Kodak diphenylguanidine was found to be  $100.0 \pm 0.1$  per cent pure.

Anhydrous acetic acid containing low concentrations of perchloric acid and acetic anhydride was prepared as follows: 20 cc. of 10.1 M  $(\text{CH}_3\text{CO})_2\text{O}$  were diluted to 1 liter with acetic acid (C.P.) and 10 cc. of acetuous 1.095 M  $\text{HClO}_4$  (and 3.00 M  $\text{H}_2\text{O}$ ) obtained by dilution of 70 per cent  $\text{HClO}_4$ . After 3 days, 3 or 6 cc. samples of this solution were added to accurately weighed 100 mg. samples of anthranilic acid, previously standardized by  $\text{HClO}_4$  titration, and, after 3 hours standing in the dark, the resulting solutions were titrated. According to the data obtained the water content of the acetic acid used was 0.09 M and the mixture prepared from it was 0.01 M in  $\text{HClO}_4$  and anhydrous, with a content of 0.085 mole of acetic anhydride per liter. This mixture was used to prepare anthranilic acid solutions (cf. Sakami and Toennies (3)) in the required concentrations as needed (allowance being made for the acetic anhydride present in calculation of the anticipated concentrations).

The acetylation mixture was prepared by mixing equal volumes of approximately 0.5 M anhydrous acetuous solutions of perchloric acid (cf. Sakami and Toennies (3)) and of acetic anhydride. This mixture is highly hygroscopic but otherwise appears to be quite stable.<sup>1</sup>

<sup>1</sup> We have found it convenient to store and use this and the  $\text{HClO}_4$  titrating solution in the closed system of a pressure-filled, Schilling design, automatic micro burette assembly (manufactured by Acc Glass, Inc., Vineland, New Jersey). The burette, subdivided into 0.02 cc. units, delivers 10 cc. It is sealed at the top against

*Example*

Adequate amounts of the amino acids to be analyzed are dried to constant weight at 100° and their perchloric acid equivalent weight is determined at least in duplicate. The average precision of these determinations was  $\pm 0.2$  per cent.

Two 50 cc. glass-stoppered Pyrex Erlenmeyer flasks and one 3 cc. pipette are required for each determination (sample or blank) and one 15  $\times$  25 mm. open specimen vial for each sample. In addition one 2 cc. and one 4 cc. pipette and two Erlenmeyer flasks are needed for the anthranilic acid. Flasks, vials, and pipettes are dried overnight at 100°, and the flasks and vials are allowed to cool in a desiccator over calcium chloride. Samples of approximately 0.3 mm, or in case of dihydroxy compounds one-half of this amount, are weighed into the vials with a precision of about 0.1 per cent. After each vial is placed in a flask, the latter are dried again for 1 hour, and after cooling all flasks are weighed (precision about 0.2 mg) and then kept overnight in a desiccator.

The next morning 4 cc. portions of the acetylation mixture are added to each sample and to each flask intended for a blank determination, followed by weighing. In this and all subsequent manipulations involving the acid mixture exposure to atmospheric humidity is kept at a minimum. We have run in a single experimental group as many as sixteen samples (eight substances in duplicate), four complete blanks, and two anthranilic acid blanks. By gently swirling one makes sure that the samples are dissolved and the solutions are well mixed. The stoppered flasks are then kept in a desiccator for 2 hours. In the meantime 4 cc. portions of the anthranilic acid solution are pipetted into flasks and weighed. 3 cc. of the reaction mixture are then transferred by pipette from the first set of flasks to the anthranilic acid flasks, followed again by weighing. After these mixtures are left in the dark for 3 hours, they are titrated with perchloric acid. Those solutions which may be expected to require the largest titrations are titrated first, and by interrupting the subsequent titrations at the beginning of the color change in order to add a proper amount of acetic acid care is taken that at the end of the titrations the

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the atmosphere by a thistle tube with two 25 cc. bulbs, partly filled with the solution used, and a calcium chloride tube attached to the outer end of the thistle bulb. When the assembly is not in active use, the calcium chloride tube is kept stoppered. At the lower end a drying tube, filled one-half with soda lime (near bulb) and one-half with calcium chloride, is inserted between the pressure bulb and the air inlet tube. When not in use the tube is shut off from the assembly by a pinch cock. While for the titrating solution a very fine burette tip is desirable, the acetylation mixture, owing to its higher viscosity, requires a wider tip, approximately of 1 mm. diameter, or permitting delivery of 10 cc. in 1 minute.

volume of all solutions (except anthranilic acid blanks) is the same within approximately 1 cc. In the titrations we have registered three or four different end-point shades (green, green-yellow, yellow-green, yellow) and then based the calculations throughout on that particular shade (yellow-green) which gave the most concordant results. The choice may vary for different individuals. For illumination we have found bright diffuse daylight best, and far superior to the light of either incandescent or fluorescent lamps. The examples given in Table I show, in an abbreviated fashion, the type of data obtained. The steps involved in calculating the final results are as follows:

*Steps in Calculation of Results; Cf. Table I*—From Determinations 5 and 6 the effective concentration of the anthranilic acid solution is calculated, 0.402 microequivalent per mg.

From Determinations 3 and 4 the effective total concentration of acids<sup>2</sup> in the acetylation mixture is calculated, 0.423 microequivalent per mg.

The precision of the blanks (Determinations 3 and 4) is tested as follows: If both are free of any error, the difference between them with respect to total acids (acetylation mixture plus titration value) should equal their difference with respect to total bases (anthranilic acid). In the present case  $(3184.4 - 3151.7) \times 0.423 = 13.8$  microequivalents;  $(3.32 - 3.46) \times 0.1030 \times 10^3 = -14.4$  microequivalents. Total acids of difference  $13.8 + (-14.4) = -0.6$  microequivalent. Total bases of difference  $(4200.5 - 4207.1) \times 0.402 = -2.7$  microequivalents. Total acids minus total bases (of difference) = 2.1 microequivalents. This value represents an error, in terms of titration, of 0.02 cc. and may be considered as falling within the normal limits of error (about 0.05 cc.). By applying this pairing test to two or more blank determinations any single faulty determination can readily be eliminated.

The results of the available complete blanks are averaged; in the present case, 4203.8 mg. of anthranilic acid solution, 3168.1 mg. of reaction mixture, and 3.39 cc. for titration.

The further steps are illustrated in Table I. In line (p) the amount of acetylation mixture contained in the 3 cc. sample added to the anthranilic acid solution is calculated. Line (q) gives the corresponding blank value. The difference between substance and blank determination (r) is converted into microequivalents of acids (s). Similarly the titration difference between substance and blank determination (o) is converted into microequivalents of acids (u). Line (v) gives the total difference in acids.

<sup>2</sup> Both  $\text{HClO}_4$  and  $(\text{CH}_3\text{CO})_2\text{O}$  may be considered as "acids" because both "neutralize" anthranilic acid (the latter being a misnomer in the acetous system because it functions as a base), one by salt formation, the other by acetylation.

TABLE I  
Illustration of Data and Calculations in Hydroxy Determinations

(a) Determination No. . .	1	2	3	4	5	6
(b) Substance . .	<i>dl</i> -Threonine		Complete blanks		Anthranilic acid blanks	
(c) HClO <sub>4</sub> titration, theoretical, <i>microequivalents per mg.</i>	8.398					
(d) HClO <sub>4</sub> titration, found, <i>microequivalents per mg.</i>	8.504 ± 0.022					
(e) Sample, <i>mg.</i> . . .	36.98	35.48				
(f) " <i>microequivalents</i> . . . .	314.5	301.7				
(g) Acetylation mixture, <i>mg.</i> . . .	4300.8	4259.0				
(h) Total reaction mixture, <i>mg.</i>	4337.8	4294.5				
(i) Anthranilic acid solution, <i>mg.</i> . . .	4213.5	4212.2	4200.5	4207.1	2120.3	2121.6
(j) Reaction mixture, 3 cc., <i>mg.</i> . . .	3154.8	3164.8	3184.4	3151.7		
(k) Titration, 0.1030 N, 25°, green, cc.	7.66	7.42	3.17		8.08	8.10
(l) Same, green-yellow, cc.	7.78	7.54	3.26	3.39	8.18	8.18
(m) Same, yellow-green, cc.	7.88	7.66	3.31	3.45	8.24	8.24
(n) Same, yellow, cc.	7.94	7.73	3.36	3.49	8.29	8.31
(o) " yellow-green, corrected, cc.	7.91	7.69	3.32	3.46	8.28	8.28
(p) (j)/(h) × (g), <i>mg.</i>	3127.9	3138.7				
(q) Reaction mixture (average blank), <i>mg.</i>	3168.1	3168.1				
(r) (p) - (q), <i>mg.</i>	-40.2	-29.4				
(s) (r) × 0.423, <i>microequivalents</i>	-17.0	-12.4				
(t) (o) - 3.39 (average blank), cc.	4.52	4.30				
(u) (t) × 0.1030 × 10 <sup>3</sup> , <i>microequivalents</i>	465.5	443.0				
(v) (s) + (u), <i>microequivalents</i>	448.5	430.6				
(w) (i) - 4203.8 (average blank), <i>mg.</i>	9.7	8.4				
(x) (w) × 0.402, <i>microequivalents</i>	3.9	3.4				

TABLE I—Concluded

(a) Determination No.....	1	2	3	4	5	6
(b) Substance.....	<i>dl</i> -Threonine		Complete blanks		Anthranilic acid blanks	
(y) (j)/(h) × (f), micro-equivalents.....	228.7	222.3				
(z) (x) + (y), microequivalents.....	232.6	225.7				
(aa) (v) - (z), microequivalents.....	215.9	204.0				
(bb) (aa)/((c) × (j)/(h)), microequivalents per mg.....	8.028	7.837				
(cc) 100 (bb)/(c), %.....	95.6	93.3				
(dd) Base value found, % of theoretical.....	101.3 ± 0.3					
(ee) Hydroxy value found, % of theoretical.....	94.5 ± 1.2					

Similarly, from the difference in anthranilic acid (*w*, *x*) and amino acid (substance analyzed) (*y*) the total difference in bases is obtained (*z*). The resulting apparent excess of acids over bases (*aa*) must be attributed to the presence, in the sample, of pseudobases, *i.e.* groups which are not titrated with  $\text{HClO}_4$ , but which "neutralize" "acids" in the acetylation mixture, a condition fulfilled by the reaction of hydroxy or analogous groups with acetic anhydride. The amount of such groups is now related to the amount of substance involved (*bb*, *cc*), and in the final two lines the values found for bases ( $-\text{NH}_2$ ) and for pseudobases ( $-\text{OH}$ ) are expressed as percentages of the theoretical base value of the substance under analysis.

## DISCUSSION

The results obtained in the manner described are summarized in Table II. The average precision (average deviation) of the hydroxy determinations is about  $\pm 1$  per cent. That this figure represents average accuracy as well may be assumed for the following reasons. Among the four hydroxyamino acids investigated in the preceding study (Sakami and Toennies (3)) the slowest hydroxy reaction was shown by tyrosine. Therefore, tyrosine was used as the object of experimentation in developing the present method. It was found that under the present conditions (at least 0.125 *M* free  $\text{HClO}_4$  and 150 per cent excess acetic anhydride) a reaction period of  $\frac{1}{2}$  hour is not sufficient, a value of  $97.7 \pm 0.7$  per cent (of the corresponding  $-\text{NH}_2$  value) being obtained, while after 1, 2, and 3 hours of reaction values of  $100.0 \pm 0.5$ ,  $99.3 \pm 0.6$ , and  $98.9 \pm 0.3$  per cent resulted,

and while blank determinations run for 1 hour or for 3 hours gave identical figures. These data, each value representing the average of triplicate determinations, led to the adoption of 2 hours as the standard reaction period. The conditions for the second reaction (with anthranilic acid)

TABLE II  
*Results of Hydroxy Determinations on Amino Acids*

Substance	Mol. wt.	Basic group titrated by HClO <sub>4</sub>	Apparent hydroxy groups found
		<i>per cent of theoretical</i>	<i>per cent of theoretical basic groups</i>
<i>dl</i> -Serine I. ....	105.1	98.1 ± 0.1	95.0 ± 1.3
" II. ....	105.1	99.2 ± 0.1	99.2 ± 1.0
<i>dl</i> -Threonine I. ....	119.1	101.3 ± 0.3	94.2 ± 1.2
" II. ....	119.1	99.6 ± 0.2	102.5 ± 1.0
<i>dl</i> -Allothreonine. ....	119.1	98.7 ± 0.2	96.7 ± 2.3
<i>l</i> -Tyrosine I. ....	181.1	98.0 ± 0.2	96.3 ± 1.7*
" II. ....	181.1	99.3 ± 0.0	98.6 ± 0.6*
<i>dl</i> - <i>p</i> -Hydroxyphenylglycine. ....	167.1	99.8 ± 0.2	95.1 ± 0.8
<i>dl</i> -Dihydroxyphenylalanine. ....	198.1	99.7 ± 0.2	195.0 ± 0.6
<i>l</i> -Hydroxyproline. ....	131.1	100.3 ± 0.2	98.2 ± 1.0
O-Acetyl- <i>l</i> -hydroxyproline. ....	173.1	99.3 ± 0.1	-0.2 ± 1.6
N-Acetyl- <i>l</i> -hydroxyproline. ....	173.1	(99.6†)	90.2 ± 0.4
<i>l</i> -Tryptophane. ....	204.1	99.9 ± 0.0	100.8 ± 0.2
<i>l</i> -Cysteine. ....	121.1	93.3 ± 0.2†	85.8 ± 0.7
<i>l</i> -Cystine. ....	240.2	97.4 ± 0.2	-36.7 ± 0.0
<i>dl</i> -Methionine. ....	149.1	99.7 ± 0.0	0.8 ± 0.0
<i>l</i> -Arginine hydrochloride. ....	210.1	101.2 ± 0.1§	103.2 ± 0.4
" .....	174.1	77.1 ± 0.2   ¶	64.3 ± 0.9
<i>l</i> -Citrulline. ....	175.1	93.4 ± 0.9  **	-2.8 ± 0.1
<i>l</i> -Histidine hydrochloride + 1H <sub>2</sub> O. ....	209.6	102.5 ± 0.1§	97.5 ± 4.5
<i>dl</i> -Alanine. ....	89.1	99.8 ± 0.1	-1.4 ± 1.1††
<i>l</i> -Glutamic acid. ....	147.1	100.2 ± 0.2	-2.7 ± 0.1

\* Three determinations.

† Acidity, determined by titration with aqueous alkali.

† The results of iodometric analysis (Lavine (2), Toennies and Lavine (6)) corresponded to 94.7 per cent cysteine and 4.4 per cent cystine.

§ Of 1 equivalent per molecule.

|| Of 2 equivalents per molecule.

¶ Had lost 16 per cent on drying.

**\*\* Titration difficult (weak basicity of ureido group).**

†† Four determinations.

have been studied in the preceding paper. In connection with the present work it was found that if the anthranilic acid solution is pipetted into the tyrosine-containing acetylation mixture the final titration is about 0.01 milliequivalent higher than when the latter is pipetted into the former,

while identical values result when the acetylation mixture contains no amino acid (blanks). For the present it remains undecided whether or not this behavior can be explained by azlactone formation, a reaction in which 2 molecules of acetic anhydride disappear for each amino group.

The compounds listed in Table II were, except for a few of our own preparations, obtained from different commercial sources. In addition to the evidence for varying degrees of purity several observations require additional comment. Hydroxyl-free compounds found to consume acetic anhydride are tryptophane, cysteine, and arginine. The apparent reaction of the histidine salt is due to its water of crystallization. The reactions of tryptophane and cysteine presumably involve the formation of the unknown N-acetylindyl- $\beta$ -alanine and S-acetylcysteine. The negative value obtained in the case of cystine seems to result from an oxidative action of perchloric acid, in which the latter is reduced to hydrochloric acid with formation of sulfuric acid, and perhaps cysteic acid, from cystine. The presence of these added strong acids (sulfate was shown to be present) would account for the lowering of the perchloric acid titration value which is the cause of the negative "hydroxy" figure; and the presence of chloride, to the extent of 0.9 to 1.0 milliequivalent per mm of cystine, was actually established in the reaction mixture. This behavior of cystine is not entirely surprising, because similar observations were recorded by one of us some years ago (Toennies and Lavine (5)). It is of interest, however, that no other amino acid has shown evidence of being oxidized by perchloric acid under the prevailing conditions, and especially that the sulfur of cysteine seems to become protected against oxidation by being acetylated (the cysteine reaction mixtures contained some chloride and sulfate; however, the figures obtained suggest that S-acetylation represents the main reaction) and that the sulfur of methionine likewise proves entirely stable. The opposite relation between methionine and cystine with respect to oxidizability was observed when aqueous hydrogen peroxide is the oxidant (Toennies and Callan (4)).

The experiments with arginine (probably containing carbonate) and its hydrochloride indicate that a non-basic group is undergoing acetylation. Since the reaction is not shown by citrulline, it may be surmised that the imino group is being acetylated and that the ureido group of citrulline does not isomerize into  $\text{HO}-\text{C}(=\text{NH})-\text{NH}-$ .

The authors are indebted to Miss Fernanda Misani for her diligent help in performing the perchloric acid analyses of the amino acids.

#### SUMMARY

Hydroxy and analogous ( $-\text{NH}-$  of tryptophane,  $=\text{NH}$ , and, imperfectly,  $-\text{SH}-$ ) groups can be determined in dry amino acids by an acetous

titrimetric method based on the acid-catalyzed acetylation of these groups by acetic anhydride. The evidence obtained indicates that the reaction employed may furnish a way for preparing the unknown S-acetylcysteine, the imino-N-acetyl derivative of arginine, and the indole-N-acetyl derivative of tryptophane. Among the amino acids studied cystine shows an exceptional behavior in that it reduces perchloric acid under the prevailing conditions. Diphenylguanidine has been found suitable as a primary standard for aceteous perchloric acid.

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# A COMPARISON OF THE HEPARINS OF VARIOUS MAMMALIAN SPECIES

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It has been previously reported by Jaques and Waters (1) that heparin isolated from dog tissue is not identical with heparin obtained from beef tissue. While at present chemically indistinguishable, dog heparin possesses a biological activity  $2\frac{1}{2}$  times greater than beef heparin. Jaques (2) has reported that heparin from pig and sheep tissue is also different, both showing lower biological activity than that from beef. Charles and Todd (3) have reported the results of various studies on beef heparin. Heparin isolated from various beef tissues and also fractionated in different ways was always completely recovered as a crystalline barium salt of fixed potency. Similar results have been obtained with heparin from dog tissues (Jaques and Waters) and from pig tissue. As reported here, the heparins of different species show slight but definite differences in the optimal conditions necessary for crystallization; so that it would be possible to separate them by crystallization. Since no fractionation of either beef heparin or dog heparin was accomplished by repeated crystallization and as isolation of heparin from the four species has always yielded heparin of the activity characteristic of the species, each is to be regarded as a chemical entity and not as a mixture of closely related substances. The heparins of different species evidently possess differences in chemical structure.

From the tissues of each species so far examined, the heparin was isolated as the crystalline barium salt by the procedure of Charles and Scott (4). In the experimental part is described the isolation of the particular lots of the different heparins submitted to complete analyses. Under the microscope all the samples gave the same appearance, showing the typical rosettes and sheaves observed with beef heparin. Fig. 1 shows the crystals of pig heparin. The various salts of heparin are isomorphous and hence a mixed barium-ammonium salt is frequently obtained. The analytical data reported in Table I show that the various samples were mixed barium-ammonium salts but the determination of the amount of barium and ammonium bound demonstrates that while the different samples vary in the proportions of the different bases bound, this bears no relation to biological potency. Each type of heparin was recrystallized before being submitted to analysis and testing. These data have been used to calculate

the values for the free acid, as shown in Table I. When calculated for the free acid, the biological potencies give a simple series, 1:2:5:10, dog heparin having 10 times the potency of sheep heparin. The biological assays were conducted by a modification of the Howell method (5), in which the potency of the unknown was compared directly with that of a standard sample of the crystalline barium salt of beef heparin to which the value of 100 units per mg. had been assigned. The sulfur and nitrogen contents calculated for the free acid are identical within the experimental error. The average sulfur content = 14.3 per cent, the average nitrogen content = 2.7 per cent. The nitrogen values differ considerably. Since, however, these represent the difference between the total nitrogen and ammonia nitrogen after correction for the water and barium content of the samples, the differences shown must be considered as being within the ex-



FIG. 1. Crystalline barium salt of pig heparin. Magnification,  $\times 400$  Photograph, courtesy of Dr. D. H. Hamly, Department of Botany.

perimental error. Difficulties are encountered in determining the optical activity since this is low, and with high concentrations of heparin the solution interferes with the measurement. However, no difference in optical rotation was observed.

A marked chemical property of heparin is its power to combine with acids and other complex bases; e.g., the protamines (Fischer). Heparin samples were titrated with the protamine salmine by

adding different amounts of heparin solution varying amounts of protamine solution to give a total volume of 0.2 cc. To each mixture at  $37^\circ$ , 1 cc. of dog blood was added and the mixture found which had no effect on the clotting time. The number of mg. of protamine required to neutralize 1 mg. of heparin from the different species are shown in Table I. Within the limits of accuracy of the method, the protamine-combining power of each heparin is the same and does not vary with the biological

activity. The combining power of heparin was also tested by the use of azure dyes. The heparin salt of certain samples of toluidine blue is red in color, in contrast to the blue of the original dye (Jorpes, Holmgren, and Wilander (7)). Hence this property can be used to estimate heparin. The results are shown in Table I. On a weight basis the different heparins show the same combining power with toluidine blue. From the results of the protamine and toluidine blue tests it is evident that there is no difference between the different heparins in their base-binding power, in contrast to the different biological potencies observed. This implies not

TABLE I  
*Comparison of Crystalline Barium Salts of Various Heparins*

	Dog	Beef	Pork	Sheep
Potency, units per mg.	240	100	44	23
Water of crystallization, %*	7.0	15.2	8.8	7.0
Ash, %	29.07	33.0	37.06	28.8
N, %†	3.76	1.93	3.13	4.77
NH <sub>3</sub> -N, %	1.43	0.00	1.36	2.56
S, %†	10.76	10.70	10.36	11.62
Ba, %†	22.0	22.7	25.05	18.15
[α] <sub>D</sub> <sup>20</sup> , degrees	+56	+55	+53	+53
Protamine titer, mg. per mg. heparin	2.1	1.9	2.2	2.3
Toluidine blue assay	92	92	106†	97
Calculated for free acid				
Potency, units per mg.	335	152	66	31
S, %	14.0	14.4	14.1	14.7
N, %	3.0	2.6	2.4	2.8

\* Loss in weight on drying *in vacuo* over P<sub>2</sub>O<sub>5</sub> (at pressure 0.001 mm. of Hg and at 100°).

† Determined on the anhydrous material dried in this way. All other determinations were made on material dried *in vacuo* over CaCl<sub>2</sub> at room temperature. The protamine titer and toluidine blue assay were conducted with the sodium salt.

‡ Different dye sample.

only the same number of acid groups but also that the various heparins possess the same affinity for protein. In this connection Jaques and Charles (5) have reported that when heparins are assayed for their effect on isolated stages of the clotting system, *i.e.* their antithrombin or antiprothrombin activity, the differences between the heparins of the different species are not quite as marked as when observed with whole blood.

The heparins were also compared for their biological action *in vivo*. The effects of intravenous injections of beef heparin in dogs have been previously reported (8) and the limits established for the standard dose of

30 units per kilo. This dose of the various heparins was administered to a dog of 8.6 kilos and the clotting time followed (Table II). Similar injections of beef heparin which served as controls were also given. It can be seen that the sheep, dog, and pig heparin gave the same curves as the control beef heparin, as judged particularly by the maximum clotting time and time required for the clotting time to return to normal, although the actual amounts of heparin given were from 1.3 mg. of dog heparin to 13 mg. of sheep heparin. The variations shown are within the normal range and the clotting time was back to normal in all cases in 45 minutes.

TABLE II

*Action in Vivo of Heparin from Different Species*

The heparin was given intravenously to a dog of 8.6 kilos under amytal anesthesia. The clotting times were determined by the congluometer (Murray *et al.* (9)). All readings are measured in minutes.

Time from injection	Clotting times					
	Beef	Sheep	Dog	Beef	Pig	Beef
	Heparin dosage					
	2.6 mg., 30 units per kilo	13 mg., 30 units per kilo	1.3 mg., 30 units per kilo	2.6 mg., 30 units per kilo	8.6 mg., 30 units per kilo	5.2 mg., 60 units per kilo
	6	5	3	3	2	2
5	11	8	>45	>40	>45	18
10	19	17	26	9	>45	
15	13	21	9	21	6	24
25	9	11	5	4	6	
35	3	3	7	2	8	13
45	5	3	3	2	3	16
60						9
75						5
Peak value.....	19	21	>45	>40	>45	
Duration of effect, min.....	35	35	45	35	45	75

In contrast, when double the amount of beef heparin was given, it took twice as long for the clotting time to return to normal, and it has been previously shown that the period of time in which heparin remains in the circulation is directly proportional to the amount of heparin administered. It is of interest that the heparins disappear from the circulation at a rate proportional to their biological activity. This is of both practical and theoretical importance. It indicates that it is unlikely that a longer acting heparin can be obtained by using other species as a source of heparin, and it also suggests that the chemical difference between the heparins is one which affects their destruction *in vivo* (presumably by heparinase).

The results reported demonstrate that there are different heparins in different mammalian species, as shown by biological assay. The chemical basis for this difference is not known. The elementary analysis, optical rotation, and protein-binding power were found to be the same with all heparins. The difference, therefore, is probably not due to differences in the sulfur, nitrogen, carbohydrate, or acid components of heparin. The fact that their biological activities form a simple series suggests that the factor involved is one that can be varied in some simple manner. Two possible factors are (1) some substituent group, (2) variation in the length of the chain. It has been suggested that heparin and the mucoitin-sulfuric acids have a long chain structure similar to starch and cellulose. The fundamental unit for this chain, as determined by Charles and Todd, is a four hexose unit, containing two glucosamine, two glycuronic acid, and five sulfuric acid groups. The experiments of Bergström (10) and of others on the anticoagulant action of sulfonated polysaccharides indicate that in these compounds at least anticoagulant activity is a function of the length of the chain.

#### EXPERIMENTAL

*Isolation of Pig Heparin*—100 pounds of fresh pork lung were treated by the method of Charles and Scott (11) and 63.5 gm. of crude heparin (potency <0.25 unit per mg.) were obtained. This was dissolved and treated with carbonate, charcoal, and Lloyd's reagent as described by Jaques and Waters. On precipitation with 2 volumes of alcohol, this gave 3.0 gm. of purified heparin (10 units per mg.). This was dissolved in 25 cc. of water by adding aqua ammonia until strongly alkaline to litmus; 7.5 cc. of 20 per cent ammonium carbonate were added and the mixture heated to 65°, cooled, and centrifuged. The supernatant was acidified with acetic acid, and sulfate removed by the cautious addition of 50 per cent barium acetate solution, followed by centrifugation. To the supernatant were added 3.2 cc. of 50 per cent barium acetate solution. A semicrystalline precipitate appeared immediately, with a potency of 27.5 units per mg. This was dissolved in 5 cc. of water, the barium removed with ammonium carbonate, and the heparin precipitated with benzidine. After removal of the benzidine the solution was acidified with acetic acid and precipitated with 2 volumes of alcohol (731 mg. at 35 units per mg.). The precipitate was dissolved in water and, after treatment with 20 per cent ammonium carbonate as before and acidification, was diluted to a total volume of 30 cc. (26 mg. of heparin per cc.); 7.8 cc. of 10 per cent barium acetate and 8.0 cc. of glacial acetic acid were added to the solution at 65°. On cooling, the precipitate came out in the usual sheaves and rosettes. The yield was 540 mg. (44 units per mg.), 23,800 units.

*Isolation of Sheep Heparin*—100 pounds of sheep lung were treated similarly. The crude heparin showed no potency. After purification as above and treatment with benzidine, 9.0 gm. were obtained, with a potency of 6.9 units per mg. They were dissolved in 35 cc. of water, treated with 10.5 cc. of 20 per cent ammonium carbonate, diluted to 250 cc., and 46 cc. of 10 per cent barium acetate and 53 cc. of glacial acetic acid added. 3.6 gm. of crystalline barium salt (23 units per mg.) were collected. The crystals were very fine and small. In order to obtain crystals of normal size, the heparin was recrystallized. 3.4 gm. were dissolved in 17 cc. of water and after being treated with 5.1 cc. of 20 per cent ammonium carbonate were diluted to 75 cc. (45 mg. per cc.) and 15 cc. of 20 per cent barium acetate and 17 cc. of glacial acetic acid added to the solution at 65°. On cooling, the precipitate came out in the usual rosettes and sheaves. Yield, 2.4 gm. (23 units per mg.), 55,200 units.

*Isolation of Dog Heparin*—The livers of three large dogs (2 pounds of tissue) yielded 5.9 gm. of crude heparin, containing 3.5 units per mg. On purification, this gave 1.5 gm. (25 units per mg.) of material. This was dissolved and precipitated as the barium salt by adding an excess of barium acetate. The 200 mg. obtained were dissolved in 10 cc. and the barium removed with ammonium carbonate. The total volume was made up to 17 cc. (6 mg. of heparin per cc.) and 2.8 cc. of 10 per cent barium acetate and 2.6 cc. of acetic acid added to the hot solution. On cooling this gave small but very perfect crystals. The yield was 94.5 mg. (250 units per mg.).

It has been the experience of the authors with beef heparin that the final concentration of heparin is a critical factor for good crystallization. Concentrations higher or lower than 30 mg. per cc. result in poor crystals with poor yields. As shown above, the concentration of sheep heparin had to be increased to 45 mg. per cc., that of dog heparin decreased to 6 mg. per cc. to obtain a crystallization comparable in gross and microscopic appearance and yield with that of the beef heparin. This has been found consistently with such crystallizations.

#### SUMMARY

Heparin has been isolated in the form of its crystalline barium salt from dog, beef, pork, and sheep tissue. The heparins of these species show marked differences in biological activity, their anticoagulant potencies being in the order 10:5:2:1. It is suggested that this difference in potency may be due to differences in chain length of the molecule. No chemical difference has thus far been detected between these different heparins. They contain the same percentage of sulfur and nitrogen and possess the same optical rotation and combining power for proteins.

The authors are greatly indebted to Professor C. H. Best for his kindly interest and encouragement in the problem.

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# AN ENZYMATIC CONVERSION OF RADIOACTIVE SULFIDE SULFUR TO CYSTEINE SULFUR\*

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An enzyme system that forms hydrogen sulfide from cysteine was described in an earlier paper (1). Pyruvic acid and ammonia were other main products of the reaction. The work reported in the present paper was carried out to determine whether or not the formation of hydrogen sulfide could be reversed and the formation of cysteine sulfur from sulfide sulfur demonstrated. It seemed clear that if the reversal took place it could be demonstrated by carrying out the enzyme reaction on cysteine in the presence of added hydrogen sulfide containing radioactive sulfur. If cysteine is reformed from hydrogen sulfide during the reaction, then the cysteine present after a part of the initial compound has been converted to the above products should contain radioactive sulfur. The work reported below indicates that such is the case.

## EXPERIMENTAL

The radioactive sulfur ( $S^{35}$ ) was very kindly supplied to us as barium sulfate by the Radiation Laboratory of the University of California. It was reduced by heating with a mixture of carbon, aluminum powder, and sodium bicarbonate (2). After the mixture was acidified, the  $H_2S$  was driven over into cadmium acetate solution by a stream of nitrogen. It was released from the cadmium as needed by the addition of  $HCl$  and passed into sodium hydroxide. The concentration of  $Na_2S$  was determined by adding excess iodine and titrating back.

The enzyme preparations were the chloroform-treated extracts previously described (1). They were buffered at pH 7.6 with phosphate buffer in an atmosphere of nitrogen, made approximately 0.01 M with cysteine and 0.001 M with sodium sulfide containing radioactive sulfur. The mixtures were incubated at  $37^\circ$  until tests showed that the nitrogen of one-quarter or more of the cysteine had been converted to ammonia. The hydrogen sulfide present at this time was recovered by sweeping out with

\* The authors are indebted to the Buhl Foundation for a research grant in support of the present investigation. Contribution No. 462 from the Department of Chemistry, University of Pittsburgh.

A preliminary account of these experiments was presented before the Thirty-sixth annual meeting of the American Society of Biological Chemists at Boston, April 2, 1942 (*Federation Proc.*, 1, pt. 2, 134 (1942)).

nitrogen and the proteins were then precipitated, usually by the addition of 1 volume of alcohol. The filtrate was treated with barium acetate, the resulting precipitate was removed by centrifugation, and the solution, plus the washings from the protein and barium precipitates, was evaporated to dryness under reduced pressure. The dry material was extracted with 1.0 N HCl. Any insoluble material was discarded. The HCl solution was placed in a boiling  $\text{H}_2\text{O}$  bath and treated with  $\text{Cu}_2\text{O}$  equivalent to 8 times the cysteine added. The solution was adjusted to a pH of about 4.0 by the addition of sodium acetate and allowed to stand at room temperature for at least 40 minutes. It was then centrifuged and the precipitate was washed with cold 95 per cent alcohol, suspended in water, and treated with excess  $\text{H}_2\text{S}$ . The  $\text{CuS}$  was centrifuged, washed, and discarded. The solution was aerated to remove  $\text{H}_2\text{S}$ , made approximately neutral, treated with a trace of cupric salt, and aerated until the nitroprusside reaction was negative. The pH was adjusted to approximately 4.0 and the solution evaporated to dryness under reduced pressure. The dry material was extracted with cold water to remove salts and then with 1.0 M HCl. Any soluble material was discarded. If any color was present, the acid solution was treated with norit until the color was removed. The clear solution was adjusted to about pH 4.0, treated with an equal volume of alcohol, cooled to  $0^\circ$ , and allowed to stand. The precipitate that formed represented the crude cystine. The product was recrystallized by various procedures. The one used most often was solution in hot water and precipitation by the addition of 0.5 volume of alcohol and cooling to  $0^\circ$ . Solution in dilute ammonium hydroxide followed by neutralization with acetic acid was also used, as was solution in the minimum amount of 0.1 N HCl and precipitation with alcohol. Typical hexagonal crystals were obtained from each sample.

*Measurement of Radioactivity*—The activities of the samples were measured by placing them inside a Geiger-Müller counter filled with helium at atmospheric pressure. The counter was connected to a counting rate meter of the recording type. The background averaged about 65 counts per minute. The relative sensitivity of the counter was determined before and after each measurement by measuring the activity of a uranium standard. All activities were referred to a standard counter sensitivity. The statistical probable error of the figures given below does not exceed 3.0 per cent in any case. The samples were counted in micro beakers having a diameter of 1.8 cm. They were added to the beakers either as a solution or as a suspension and then dried at  $100^\circ$  before being tested. Our starting material was a solution prepared by passing  $\text{H}_2\text{S}^{35}$  into 0.1 N NaOH, as described above. Different amounts of this solution (of suitable dilution), when placed in a series of the micro beakers and made up to the same volume with water before drying, showed activities that were accurately propor-

tional to the amount of sulfide (Fig. 1). Conversion of the sulfide to sulfate (by alkaline  $\text{H}_2\text{O}_2$  or by  $\text{HNO}_3 + \text{KClO}_3$ ) and formation of  $\text{BaSO}_4$  for counting led to activities about one-third lower, due at least in part to absorption by the  $\text{BaSO}_4$ .

Most of our determinations of the radioactivity of cystine samples have been done on the cystine itself. In order to make the results comparable to the activity of the  $\text{Na}_2\text{S}^{35}$  used as starting material, we have multiplied the cystine activity by a factor determined by measuring the effect of ordinary cystine added to  $\text{Na}_2\text{S}^{35}$  samples. The effect of adding 5 mg. of cystine to various amounts of  $\text{Na}_2\text{S}^{35}$  is shown in Fig. 1. The effect of adding the same amount of cystine to more active samples is shown in Table I. Such

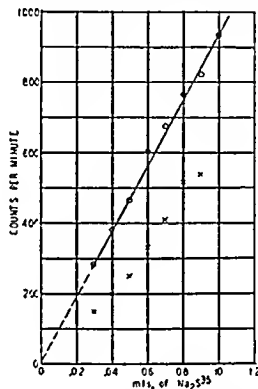


Fig. 1. Relation between the observed activity and the amount of  $\text{Na}_2\text{S}$  solution tested; and the absorption effect of cystine. O a solution prepared by passing  $\text{H}_2\text{S}$  containing  $\text{S}^{35}$  into 0.1 N  $\text{NaOH}$ . The samples were dried at  $100^\circ$  before being tested. X the same solution with 5.0 mg. of cystine added to each sample.

a correction, of course, applies only to the conditions under which the tests were made. The absorption correction factor is not directly proportional to the amount of cystine; it increases with increasing concentration under our conditions, rapidly at first, and then more slowly. The chief serious difficulty in getting consistent results is in getting a uniform layer of material for counting. For this reason comparisons should be restricted to samples that are as nearly alike as possible.

Comparisons between samples counted at different times were made by correcting for the intervening time by the formula

$$\log t = 0.00342 \times \text{time (in days)}$$

where  $t$  is the correction factor. The constant is based on the fact that the half life of  $\text{S}^{35}$  is 88 days.

*Results*—Three experiments are recorded in detail in Table II. It is

to form cysteine and then contribute some or all of this cysteine to the deproteinized solution? In the absence of added cysteine such a formation of cysteine could not be demonstrated either in the protein-free solution or in the proteins themselves, but this does not rule out such a possibility for any cysteine formed might be broken down by the  $H_2S$ -forming enzyme in the absence of added cysteine and might escape destruction in the presence of added cysteine. The present data merely prove the formation of cysteine sulfur from  $H_2S$  and do not determine the mechanism.

It may be of interest to know what proportion of the sulfur atoms of cysteine has been made radioactive by this procedure. The counter was calibrated by noting the counting rate due to a thin layer (36 mg. per sq. cm.) of naturally radioactive  $RbCl$  for which the true disintegration rate can be computed. The sulfur radiations are intermediate in energy between the two  $\beta$ -ray groups from rubidium; so this calibration should be approximately valid for sulfur also. Specific activities of about 0.1 microcurie per mg. of active cysteine were found corresponding to 1 radioactive sulfur atom in  $10^6$ . The  $H_2S$  used as starting material had a specific activity of 95 microcuries per mg., corresponding to 1 radioactive sulfur atom in  $5 \times 10^5$ .

#### SUMMARY

Cysteine and hydrogen sulfide containing radioactive sulfur have been added to an enzyme preparation which converts cysteine to pyruvic acid, ammonia, and hydrogen sulfide. When the nitrogen of part of the added cysteine had been converted to ammonia, the reaction was stopped and the remaining cysteine was isolated. It contained appreciable amounts of radioactive sulfur, thus demonstrating the formation of cysteine sulfur from sulfide sulfur under these conditions.

It is a pleasure to acknowledge our indebtedness to the Radiation Laboratory of the University of California for the generous supply of radioactive barium sulfate.

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# PHOTOELECTRIC DETERMINATION OF OXALIC ACID AND CALCIUM, AND ITS APPLICATION TO MICRO- AND ULTRAMICROANALYSIS OF SERUM

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The increased sensitivity of photoelectric methods of color measurement (9, 12) opens up a vast field of analysis in which the ordinarily great sensitivity and accuracy of iodometry may be utilized in the analysis of ultra-micro quantities of material (2, 11).

A preliminary note described the first application of iodometric reactions previously used for titrimetric work (3, 7) to the colorimetric determination of directly precipitated serum calcium (10). In the following complete report there are embodied several interim changes in procedure. The technique has been greatly simplified and made more convenient and rapid, while the range of sample which may be used has been extended, decreasing from the maximum of 1 cc. to as little as 0.02 cc. of serum for ultramicroanalysis.

The procedure, which may be carried out practically from start to finish in the same tube, and requires only ordinary laboratory materials and glassware, is simply as follows: The calcium is precipitated as the oxalate directly in diluted serum, and then isolated in pure form by alternate centrifuging and washing, first with ammonia water and then with a mixture of water, alcohol, and ether. After drying at  $110^{\circ}$ , the oxalate is dissolved in acid, and a small excess of  $\text{Ce}(\text{SO}_4)_2$  is added. An excess of KI added to the remainder of the  $\text{Ce}(\text{SO}_4)_2$  yields free iodine, the yellow color of which is measured photoelectrically. The results, standardized against known oxalate solutions, are accurate to within  $\pm 2$  per cent.

*Reagents*—The following list includes reagents for all of the analyses described.

*Ammonium oxalate, saturated and half saturated, solutions.* Ammonium oxalate, analytical reagent grade, is dissolved to saturation (about 3.5 per cent) at room temperature. The half saturated solution is prepared fresh before use.

*Ammonium hydroxide, 2 per cent.* 2 cc. of concentrated  $\text{NH}_4\text{OH}$  (26 per cent), analytical reagent grade, are diluted to 100 cc.

*Water, alcohol, ether washing mixture.* Equal volumes of distilled water, of absolute ethyl alcohol (or redistilled 95 per cent alcohol), and of ethyl ether, analytical reagent grade (or absolute, or redistilled U.S.P. ether), are

mixed. *Purity of the components of this mixture must be controlled as described on p. 255.*

*Approximately 1 N sulfuric acid solution.* 27 cc. of concentrated  $\text{H}_2\text{SO}_4$ , (sp. gr. 1.84), analytical reagent grade, are diluted to 1 liter.

*Approximately 0.5 N, 0.2 N, and 0.1 N sulfuric acid solutions* are made by dilution from the 1 N solution.

*Approximately 0.1 N ceric sulfate solution.* 29 gm. of anhydrous  $\text{Ce}(\text{HSO}_4)_4^1$  are roughly weighed and dissolved in 1 N  $\text{H}_2\text{SO}_4$  to make 500 cc. of solution. Adjustment to approximate more closely a 0.1 N solution is made as described on p. 250. For maximum stability, this solution and the dilute ones made from it should be kept in amber bottles, and otherwise protected from light (14).

*Approximately 0.005 N, 0.0035 N, 0.002 N, 0.001 N, 0.0007 N, and 0.00035 N ceric sulfate solutions* are freshly made from the adjusted 0.1 N solution. Dilutions to concentrations of from 0.005 to 0.002 N are made with 0.2 N  $\text{H}_2\text{SO}_4$ ; weaker solutions are made by dilution with 0.1 N  $\text{H}_2\text{SO}_4$ . The 0.0007 and 0.00035 N solutions are best made from the 0.0035 N solution.

*Standard 0.1 N sodium oxalate solution.* 3.3498 gm. of  $\text{Na}_2\text{C}_2\text{O}_4$ , analytical reagent grade, are dissolved in 52 cc. of 1 N  $\text{H}_2\text{SO}_4$  and added water, to make 500 cc. of solution. Kept in an amber bottle, this solution is stable for at least 6 months.

*Standard 0.0025 N, 0.001 N, 0.0005 N, 0.00025 N, and 0.0002 N sodium oxalate solutions* are freshly prepared from the 0.1 N solution by one or two dilutions with water.

*Approximately 1 per cent and 0.5 per cent potassium iodide solutions* are freshly prepared for use by dissolving 1.0 or 0.5 gm. of KI, analytical reagent grade, in 100 cc. of water. When tested with starch they should show no trace of free iodine.

*Ethyl alcohol, 95 per cent.* This is filtered through two layers of ashless filter paper on a Buchner funnel.

*2 per cent and 1 per cent (Lintner's soluble) starch solutions.* The 2 per cent solution is prepared anew every 2 weeks, as previously described ((8) p. 406). The 1 per cent solution is prepared freshly for use, by dilution with water.

### Procedure

The general procedure for the analysis of calcium in serum is described in the following. An outline of the quantitative and other specific details

<sup>1</sup> This salt, obtainable (in purity about 92 per cent) from the G. Frederick Smith Chemical Company, is preferable to anhydrous  $\text{Ce}(\text{SO}_4)_2$ . The bisulfate dissolves easily and completely, whereas the normal sulfate goes into solution slowly and with difficulty.

of steps followed in the analysis of 1.0, 0.5, 0.2, 0.1, 0.05, and 0.02 cc. samples is given in Table I.

*Precipitation of Calcium Oxalate*—To 1 volume of serum measured (in duplicate) into 12 or 15 cc. Pyrex conical bottomed centrifuge tubes,<sup>2</sup> 5 volumes of distilled water are added and mixed.<sup>3</sup> Two *standard* samples with 6 volumes of water are prepared at the same time, *to be treated throughout the following procedure simultaneously with, and in the same way as, serum samples.* 1 volume of saturated ammonium oxalate is added<sup>4</sup> to each tube, the contents of which are gently stirred by tapping. The tubes are covered with a rubber cap for protection against dust, and allowed to stand overnight (at least 16 hours).

*Washing and Isolation of Calcium Oxalate*—This operation is uniformly *the same for all standard and serum analyses, regardless of sample size.* The covered tubes are centrifuged for 5 minutes at 2600 R.P.M. By means of an upturned capillary, the tip of which is kept immersed, all but about 0.2 cc. of the supernatant fluid in each tube is slowly siphoned off.<sup>5</sup> Then the entire inner surface of the tube is washed with 3 cc. of 2 per cent ammonia water, slowly admitted from a pipette moved around the top of the wall. The ammonia water is mixed with the residual diluted serum by tapping the tube until upward movement of the solid calcium oxalate at the bottom *just begins.* Centrifugation and withdrawal of supernatant fluid are repeated.

The water-alcohol-ether (W. A. E.) mixture is then used for additional washings. After the addition of 1 cc., the tubes are stirred and the con-

<sup>2</sup> For analyses in which the final volume of the colored solution is 10 cc. (Table I), the centrifuge tubes used are calibrated to contain that volume, by deliveries of 10 cc. of water from an accurate pipette.

<sup>3</sup> For the measurement of 0.1, 0.05, and 0.02 cc. samples, we have found most satisfactory the pipettes described by Sisco, Cunningham, and Kirk (13) and supplied by the Microchemical Specialties Company, Berkeley, California. When these pipettes are used, the 5 volumes of water are not added to the 1 volume sample in a single pipetting, but as five (for 0.1 cc.) or four repeated 1 volume washings of the sample pipette. For the analyses of 0.05 and 0.02 cc. samples the ammonium oxalate is conveniently added as 2 drops, or 1 drop, of the half saturated solution, respectively. For a technique of obtaining serum from small volumes of blood, the reader is referred to an article by Kirk (4).

<sup>4</sup> In a critical evaluation of serum calcium procedures, the writer (Sendroy, unpublished results) has confirmed the validity of the direct precipitation first proposed by Pfibram (6), and later adapted to the analysis of smaller samples by others. Ashed serum may also be used, but in our experience the additional work involved does not seem to justify the procedure when carried out solely for calcium analyses by the present method.

<sup>5</sup> This first step is, of course, omitted in the analysis of 0.02 cc. samples, the calcium of which is precipitated in a total volume of about 0.15 cc. (Table I).



tents well mixed. An additional 3 cc. of W. A. E. mixture are added, gently mixed with the first 1 cc. portion, but with minimum disturbance of the precipitate into the upper supernatant volume; the tubes are centrifuged and the supernatant fluids withdrawn. The washing with W. A. E. mixture, centrifugation, and withdrawal of wash fluid are repeated once more.<sup>6</sup>

The tubes are placed in an oven at 100–110°, at an angle of about 15°, and completely dried in  $\frac{1}{2}$  to 1 hour. Ether vapors, sometimes detectable at the mouth of the tube, are dissipated during the subsequent steps of the procedure.

*Solution and Oxidation of Oxalate*—At this point, different volumes of known, standard oxalate solution are added to each of the two standard tubes, the handling of which is continued, as before, in exactly the same way as that of the unknown sample tubes. Dilute  $\text{H}_2\text{SO}_4$  of the concentration and volume indicated in Table I is added to all of the tubes, which are then heated for 5 minutes in a beaker of water kept below boiling. The tubes are removed and allowed to cool to room temperature, after which appropriate amounts of  $\text{Ce}(\text{SO}_4)_2$  are added. After all the fluids have been carefully and completely mixed, the tubes are covered and allowed to stand at room temperature for 30 minutes, or in a water bath at 70° for 10 minutes.<sup>7</sup>

Ordinarily, oxidation of the oxalate in the standards and in the unknown or serum samples will be carried out simultaneously. However, it is not necessary that this be done, nor that the oxalate of all of the serum samples be oxidized at the same time. The same pair of standards may be used for any group of analyses of the same type (same sample volume and analytical procedure), provided the  $\text{Ce}(\text{SO}_4)_2$  is added and color subsequently developed with KI, in all the tubes, standards and unknowns, within the period of stability of the  $\text{Ce}(\text{SO}_4)_2$ , or 3 to 4 hours. Thus, if many analyses are carried out throughout the day, a second set of standards

<sup>6</sup> The number and volume of washings necessary to render negligible the errors caused by retention of serum and excess oxalate would ordinarily cause larger errors in loss of  $\text{CaC}_2\text{O}_4$ , either mechanically as crystals, or by solution in the washing fluid, with a percentage error especially high for ultramicroanalyses. Such errors are avoided by the use of the W. A. E. washing mixture proposed by Velluz and Deschaseaux (16), who pointed out that  $\text{CaC}_2\text{O}_4$  is practically insoluble in it. Wang (17) has called attention also to its wetting property which prevents mechanical loss of crystals. The preliminary washing with 2 per cent ammonia water serves to dilute the serum + oxalate mixture without dissolving  $\text{CaC}_2\text{O}_4$ , and avoids the precipitation of serum proteins which would result were the W. A. E. mixture added directly for the first washing.

<sup>7</sup> As factors of safety, the time periods prescribed are double those found necessary for complete oxidation. When speed is not essential, the reaction at room temperature is preferable, since less manipulation and attention are required.

is prepared and the values obtained, if different, are applied to the later results.

*Photoelectric Measurement of Oxalate As Equivalent Iodine*—Prior to further treatment, each solution should be in the vessel in which color development and dilution to final volume (Table I) are carried out. This necessitates, in the case of 1.0, 0.5, and sometimes 0.1, and 0.05 cc. analyses, a transfer to volumetric flasks. For final volumes of 15 to 30 cc., S-tubes (retested and marked for uniformity of diameter) furnished for readings with the Evelyn (1) colorimeter may be suitably calibrated and used instead. To facilitate transfer, a part of the outer rim of the centrifuge tube is coated with a thin film of molten paraffin. On solidifying, this prevents loss over the rim as the solution is decanted into the receiving vessel. 4 cc. (more may be used if the final volume allows) of water are then used, in three or four portions, to wash the walls of the centrifuge tube, and to effect quantitative removal of the excess  $\text{Ce}(\text{SO}_4)_2$ -containing mother liquor.

Following transfer, KI is added to all the solutions, with the minimum of agitation necessary to mix well. After 60 seconds, dilution is made to volume with water, or, in some cases, filtered alcohol to make 40 per cent by volume, and then water. After careful mixing,<sup>8</sup> 10 cc. portions of the colored solutions are transferred to S-tubes, which are stoppered and wiped clean and dry. The yellow iodine color is read at  $25^\circ (\pm 5^\circ)$  in the Evelyn colorimeter, as previously described, with a No. 400 or a No. 586-5 filter (12). A *reagent blank*, containing  $\text{H}_2\text{SO}_4$ , KI, and alcohol when used, in concentrations exactly the same as in the standards and samples analyzed, and prepared at the same time that these reagents are added to them, is used to set the galvanometer at 100 for the base-line reading. The yellow iodine color may be read at any time within 1 hour of its development (addition of KI), but the galvanometer must be reset at 100 with the simultaneously prepared reagent blank.

*Alternative Method of Measurement of Oxalate As Equivalent Iodine*—It is preferable and advantageous to measure the yellow color of iodine rather than the blue formed on addition of starch (12). However, should the

<sup>8</sup> On addition of alcohol, a precipitate of  $\text{Ce}_2(\text{SO}_4)_3$  sometimes forms, depending on the volume of solution already present. On dilution to volume, and mixing, however, a clear solution is obtained. Solutions made to volume in centrifuge tubes are mixed by stirring with a small rod. Solutions already in the S-tubes are carefully mixed by gentle agitation, or, in the case of 30 cc. volumes, by inversion, but *not with stirring rods or contact with stoppers*. "Parafilm" covering may be used as a temporary stopper for the S-tubes, during mixing and reading. When alcoholic solutions are poured into color tubes, air bubbles may be formed, which, if allowed to remain during the readings, will lead to error. With gentle tapping, or on standing for a minute, such bubbles rise to the surface and disappear.

TABLE I

Outline of Procedures for Micro- and Ultramicroanalysis of Calcium in Serum, with Evelyn Photoelectric Colorimeter

The volumes are measured in cc.

Material	Volume of standard $\text{Na}_2\text{C}_2\text{O}_4$ solution	Volume of $\text{H}_2\text{SO}_4$ solution	Volume of $\text{Ce}(\text{SO}_4)_2$ solution	Volume of $\text{K}_1$ solution	Volume of 95 per cent filtered alcohol	$\text{H}_2\text{O}$ to final volume	Filter No.*	Yellow color curve used	Calculation constants
1 cc. serum.....	0.0025 N	0.5 N	0.005 N	1%		30	400†	C	$D = 30$
Standard.....	3.0, 1.0	2.0	2.0	1.8		30			$C = 0.333$
Reagent blank....		2.8		1.8		30			
0.5 cc. serum.....		1.0	1.0	0.9		15	400†	C	$D = 30$
Standard.....	1.5, 0.5	1.0	1.0	0.9		15			$C = 0.333$
Reagent blank....		1.4		0.9		15			
	0.001 N	0.2 N	0.002 N						
0.2 cc. serum.....		1.0	1.0	0.6	4.0	10	400†	E	$D = 50$
Standard.....	1.5, 0.5	1.0	1.0	0.6	4.0	10			$C = 0.20$
Reagent blank....		2.0		0.6	4.0	10			
			0.001 N						
0.1 cc. serum†....		1.0	1.5	0.6	4.0	10	400†	E	$D = 100$
Standard.....	1.0, 0.0	1.0	1.5	0.6	4.0	10			$C = 0.10$
Reagent blank....		1.75		0.6	4.0	10			
	0.0005 N								
0.1 cc. serum.....		2.0	1.0	1.2	8.0	20	586-5†	I	$D = 200$
Standard.....	1.5, 0.5	2.0	1.0	1.2	8.0	20			$C = 0.05$
Reagent blank....		2.5		1.2	8.0	20			
	0.00025 N								
0.05 cc. serum....		1.0	0.5	0.6	4.0	10	586-5†	I	$D = 200$
Standard.....	1.5, 0.5	1.0	0.5	0.6	4.0	10			$C = 0.05$
Reagent blank....		1.25		0.6	4.0	10			
	0.0001 N	0.1 N	0.00035 N						
0.02 cc. serum....		1.0	1.0	0.6	4.0	10	586-5†	I	$D = 500$
Standard.....	1.0, 0.0	1.0	1.0	0.6	4.0	10			$C = 0.02$
Reagent blank....		2.0		0.6	4.0	10			
		1.0 N	0.0007 N	0.5 per cent	Starch solution, 1 per cent				
0.05 cc. serum§...		2.0	1.0	0.5	0.5	25	600		$D = 500$
Standard.....	2.0, 0.5	2.0	1.0	0.5	0.5	25			

\* See foot-note 9.

† "Brighter" switch on in the Evelyn instrument.

‡ Alternative procedure when filter No. 586-5 is unavailable.

§ Alternative procedure when filters No. 586-5 and No. 400 are unavailable.

No. 400 filter, or the No. 586-5 filter and the modified form of instrument required for it, be lacking ((12) pp. 166, 167), the blue color may be developed and measured with a No. 600 filter.<sup>9</sup>

The technique followed is based on previous work (9) and is similar to that described in the preliminary note on the present method (10).

Following the transfer of solution containing excess  $\text{Ce}(\text{SO}_4)_2$  to a volumetric flask, KI is added, then water to approximately 80 per cent of volume. With the contents at  $25^\circ (\pm 1^\circ)$ , starch solution is slowly added, with gentle mixing by rotation. Water is added to volume, and 15 cc. portions of the blue solution are promptly read at  $25^\circ (\pm 1^\circ)$ , with a No. 600 filter in place. Standards are used, as for the yellow color measurements. In place of the reagent blank, however, a pure water tube may be used.

In Table I there is included an outline for the analysis of 0.05 cc. of serum with blue color readings. For the analysis of larger samples, the same technique may be used.<sup>10</sup>

#### *Calibration; Correlation of Galvanometer Readings with Iodine Concentrations*

Calibration curves are used for evaluating yellow color galvanometer readings of the Evelyn instrument, in terms of iodine concentrations. An extension of the data of Sendroy and Alving ((12) Fig. 1 and Table I), for plotting the curves of their color Systems C, E, and I used in the present method, is given in the following.

At galvanometer (per cent transmission) readings of 90, 80, 70, 60, 50, 40, 30, 25, 20, 15, and 10, the corresponding respective values of iodine in milliequivalents per liter in the color solutions are, for color System C, 0.010, 0.023, 0.039, 0.058, 0.083, 0.115, 0.158, 0.188, 0.225, 0.277, and 0.360; for color System E, 0.007, 0.016, 0.026, 0.039, 0.055, 0.076, 0.104, 0.124, 0.148, 0.184, and 0.239; and for color System I, 0.0022, 0.0048, 0.0077, 0.0111, 0.0151, 0.0200, 0.0267, 0.0308, 0.0362, 0.0433, and 0.0544.

The validity and reliability of the use of these curves for the present method are indicated below. We have reproduced the readings many

<sup>9</sup> The Corning Violet Ultra No. 586 (5 mm. thick, polished glass) filter transmits light within the range of 328 to 388 (maximum 360)  $\mu\mu$ . Our filter was made from Melt No. 156. Evelyn (Rubicon) filters No. 400 and No. 600 transmit light within the ranges 380 to 430 (maximum 400)  $\mu\mu$ , and 580 to 635 (maximum 600)  $\mu\mu$ , respectively.

<sup>10</sup> With the exception of the constant volume of 2 cc. of 1 N  $\text{H}_2\text{SO}_4$  added to all the tubes and used to dissolve the precipitated  $\text{CaC}_2\text{O}_4$ , the volumes or concentrations of reagents are multiples of the ones given in Table I. Thus, 0.2 cc. samples would require 1 cc. of 0.0028 N  $\text{Ce}(\text{SO}_4)_2$  (in 0.2 N  $\text{H}_2\text{SO}_4$ ), 0.5 and 2.0 cc. of 0.0008 N  $\text{Na}_2\text{C}_2\text{O}_4$ , 1 cc. of 1 per cent KI, 2 cc. of 1 N  $\text{H}_2\text{SO}_4$ , and 2.0 cc. of 1 per cent starch, all diluted to 100 cc. with water.

times, under the original experimental conditions ((12) Table I) and under the conditions of the present method, and would expect little deviation from them in other laboratories. However, the analyst should redetermine these calibration curves for his own use, as follows:

For Curve C, 1 to 10 cc. portions of 0.533 mM  $\text{KIO}_3$  solution + 2.4 cc. of 0.085 M  $\text{H}_3\text{PO}_4$  + 1.2 cc. of 5 per cent KI, diluted to 100 cc. with water, are read with filter No. 400; for Curves E and I, 0.5 to 7.0 cc. of 0.533 mM  $\text{KIO}_3$  and 0.5 to 7.0 cc. of 0.133 mM  $\text{KIO}_3$ , respectively, are treated with  $\text{H}_3\text{PO}_4$  and KI as above, then diluted with 40 cc. of filtered 95 per cent alcohol and water to 100 cc., for readings with filters No. 400 and No. 586-5, respectively.

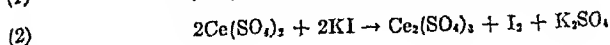
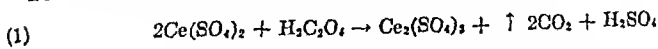
Since blue color iodine readings (with filter No. 600) are not reproducible from day to day (9), no fixed curve is applicable. However, galvanometer readings plotted semilogarithmically are straight line functions of oxalate concentrations. Such straight lines, of constant slope for a given set of conditions, are established anew for each day's work.

*Adjustment of Ceric Sulfate Stock Solution to Approximate 0.1 N Concentration*—This adjustment is not for exact standardization, but for close approximation to 0.1 N concentration; so that the more dilute solutions subsequently used for oxalate oxidation will yield colors (for iodine) falling within the desirable optimum range of readings (p. 257). Of the roughly prepared 0.1 N stock solution diluted to 20 times volume with N  $\text{H}_2\text{SO}_4$ , 1 cc. is treated with  $\text{H}_2\text{SO}_4$  and KI as for 1 cc. of serum (Table I, first line). Readings made with filter No. 400 and with reagent blank (Table I, third line), set at 100, are evaluated on Curve C. More  $\text{Ce}(\text{HSO}_4)_4$  or water is then added to the stock solution as required, to obtain a reading of about 28<sup>2</sup> at 600-fold dilution, corresponding to 0.167 milliequivalent of  $\text{I}_2$  per liter. Adjustment may be made under other conditions outlined in Table I, by using half the volume of  $\text{Ce}(\text{SO}_4)_2$  solution indicated.

Adjustment by blue color readings must be made by direct comparison with known iodate solutions (9).

### Calculations

From the reactions on which the method is based



it follows that

$$(3) \quad [\text{Ce}^{++++}] - [\text{C}_2\text{O}_4^{--}]_n = [\text{I}_2]_n$$

$$(4) \quad [\text{Ce}^{++++}] - [\text{C}_2\text{O}_4^{--}]_n = [\text{I}_2]_n$$

$$(5) \quad [\text{Ce}^{++++}] - [\text{C}_2\text{O}_4^{--}]_n = [\text{I}_2]_n$$

where brackets denote equivalent concentrations at the final volume to which the solutions are diluted for color reading, and subscripts  $s_1$ ,  $s_2$ , and  $u$  refer to *standards* and unknown sample. Subtraction of Equation 5 from Equations 3 and 4 gives two equations, the combination of which yields

$$(6) \quad [C_2O_4^-]_u = \frac{[C_2O_4^-]_{s_1} + [C_2O_4^-]_{s_2} + [I_2]_{s_1} + [I_2]_{s_2}}{2} - [I_2]_u$$

Since

$$(7) \quad \text{M. eq. Ca per liter of original sample} = [C_2O_4^-]_u \times \frac{V}{v}$$

where  $V$  = cc. volume at final dilution of color solutions, and  $v$  = cc. volume of original sample used, the working equation for any given analysis is

$$(8) \quad \text{M. eq. Ca per liter} = \left[ \frac{C + [I_2]_{s_1+s_2}}{2} - [I_2]_u \right] \times D$$

where  $C = [C_2O_4^-]_{s_1} + [C_2O_4^-]_{s_2}$ , a known constant, and  $D = V/v$ , the dilution factor, also a constant.

*Example*—In 0.2 cc. analyses (Table I), yellow color readings for triplicate serum samples were 28<sup>2</sup>, 28<sup>3</sup>, and 28<sup>1</sup>; for the standards, 18<sup>0</sup> and 46<sup>2</sup>. Values for iodine found on Curve E were 0.1095, 0.1085, 0.1105, 0.1610, and 0.0615 milliequivalent per liter, respectively. According to Equation 8, the average calcium concentration of the original serum sample was

$$\left( \frac{0.1500 + 0.0500 + 0.1610 + 0.0615}{2} - 0.1095 \right) \times \frac{10}{0.2} = 5.09 \text{ m. eq. per liter}$$

*Correction for Galvanometer Readings in Analysis of 0.02 Cc. Samples of Serum*—As is shown in a later section, under the conditions of the ultra-micro calcium analysis of 0.02 cc. samples, yellow color readings of serum involve a positive, constant, deviation in iodine values. The correction for this varies with the reading, from which it is subtracted in accordance with the scale: at reading of 15, 0<sup>1.5</sup>; 20, 0<sup>2</sup>; 30, 0<sup>3</sup>; 40, 1<sup>0</sup>; 50, 1<sup>1</sup>; 60, 1<sup>2</sup>.

*Example*—In 0.02 cc. analyses (Table I), yellow color readings for triplicate serum samples were 36<sup>1</sup>, 36<sup>1</sup>, 36<sup>1</sup>; for the standards, 23<sup>1</sup> and 55<sup>0</sup>. The former were corrected to 35<sup>1</sup>, by subtracting 1<sup>0</sup>. Values for iodine, in milliequivalents per liter, found on Curve I were 0.0230 for serum, and 0.0325 and 0.0130 for the *standards*. According to Equation 8, the calcium concentration of the original serum sample was

$$\left( \frac{0.0200 + 0.0325 + 0.0130}{2} - 0.0230 \right) \times 500 = 4.90 \text{ m. eq. per liter}$$

For blue color readings, results are calculated graphically, on a semi-logarithmic plot, with abscissa units of milliequivalents of oxalate per liter from 0 to 0.0175, and ordinate units of galvanometer readings from 10 to 100. A straight line is drawn between the points representing readings for the two standards,  $s_1$  and  $s_2$ , at concentrations of 0.016 and 0.004 milliequivalent per liter, respectively, of oxalate. Oxalate values for serum analyses, found by interpolation of their galvanometer readings on this line, when multiplied by the dilution factor  $D$  ( $= 500$ ), give directly milliequivalents of Ca per liter in the original serum samples.

*Example*—In 0.05 cc. analyses (Table I), blue color readings for duplicate serum samples were  $32^2$  and  $32^3$ ; for the standards,  $21^1$  and  $49^2$ . A straight line was drawn through the two latter values located at 0.016 and 0.004 milliequivalent of oxalate per liter, respectively. Interpolated values for the serum samples were 0.01005 and 0.01013 milliequivalent per liter, from which the average calcium concentration of the original serum was calculated to be  $0.01008 \times 500 = 5.04$  milliequivalents per liter.

#### EXPERIMENTAL

##### *Proof of Accuracy of Results*

In testing the various factors involved, during the past 2 years 177 photoelectric analyses of 146 samples of known oxalate solutions, known calcium solutions, and human sera were carried out in duplicate or triplicate. A detailed tabulation of the results being impractical,<sup>11</sup> they are listed in Tables II to IV in groups only, classified according to oxalate or calcium content. For each analysis, the average photoelectric value was compared with a reference value known, or obtained by another method. The resulting deviations of average photoelectric values from reference values were averaged for the analyses of each group. The variation in such deviations among the several analyses comprising each group was the same as the variation among the individual values of the duplicates or triplicates comprising each analysis. This is reflected in the columns indicating the extremes of deviations of photoelectric from reference values. In Tables II to IV, no distinction is made between photoelectric values obtained by yellow and blue color readings, as in our hands the results by both were the same. Amounts of calcium, from 100  $\gamma$  down to 2  $\gamma$ , were separated and washed by the present centrifugation technique equally as well as by the more tedious filtration technique for 20  $\gamma$  given in the preliminary note (10).

*Analysis of Known Oxalate Solutions*—The 0.1 N stock solutions of Na-

<sup>11</sup>These and other detailed experimental data covered in the "Experimental" section are available on request from the author.

$\text{C}_2\text{O}_4$  used as the basic standard for all determinations were compared from time to time with solutions made from Bureau of Standards sodium oxalate. In the titration of 10 to 25 cc. portions with 0.1 N  $\text{KMnO}_4$ , the results always agreed within 0.2 to 0.3 per cent.

Table II shows that photoelectric analyses of solutions varying in oxalate content from the equivalent of 100  $\gamma$  to 2  $\gamma$  of Ca were within  $\pm 2$  per cent of the known values, with extreme deviations slightly greater.

*Analysis of Known Calcium (Serum Salt) Solutions*—These solutions, approximating in content the principal inorganic constituents of serum, contained in milliequivalents per liter,  $\text{CaCl}_2$  5.0,  $\text{MgCl}_2$  3.0,  $\text{NaCl}$  154,

TABLE II

*Results of Photoelectric Analyses of Known Oxalate Solutions*

Summary of forty-six analyses (in duplicate or triplicate) of thirty known solutions of  $\text{Na}_2\text{C}_2\text{O}_4$ .

No. of analyses	Oxalate solution			Average deviation of photoelectric from known values	Extremes of deviations of photoelectric from known values
	Volume	Concentration of oxalate	Content of sample in terms of calcium		
	cc	meq per l	$\gamma$	per cent	per cent
3	1.0	5.00	100	$\pm 0.7$	-1.6, +0.4
5	0.5	5.00	50	$\pm 1.6$	-1.6, +2.0
3	0.2	3.75	15	$\pm 1.7$	-1.6, +1.9
10	0.2	5.00	20	$\pm 1.1$	-2.2, +2.0
3	0.2	6.25	25	$\pm 0.2$	-0.5, 0.0
2	0.1	2.50	5	$\pm 2.0$	-2.0, +2.0
8	0.1	5.00	10	$\pm 1.1$	-1.2, +2.0
2	0.1	6.25	12.5	$\pm 0.9$	-0.9, +0.9
6	0.05	5.00	5	$\pm 1.3$	-1.0, +3.0
4	0.02	5.00	2	$\pm 1.5$	-2.0, +2.0

and  $\text{KH}_2\text{PO}_4$  1.2. The reprecipitated  $\text{CaCO}_3$  previously used (15), or more often a crystal of Iceland spar, served as the source of calcium. Accurately weighed amounts were dissolved in the minimum amount of hydrochloric acid, to make a 25 mm stock solution. At intervals of a week or so, 10-fold dilutions in  $\text{CO}_2$ -saturated water were prepared for analytical work, by mixture with portions of stock solutions of the other salts.

Table III shows that photoelectric analyses of serum salt solutions varying in calcium content from 100  $\gamma$  to 2  $\gamma$  were also within  $\pm 2$  per cent of the amounts known to be present. The added difficulty in handling the precipitated calcium oxalate, over and above that involved in the oxalate analysis, is reflected in the extremes of deviation between photoelectric and reference values (about  $\pm 3.0$  per cent).



*Analysis of Serum Samples*—Specimens of human serum from clotted or defibrinated blood were obtained from ambulant patients with minor ail-

TABLE III

*Results of Photoelectric Calcium Analyses of Known Salt Solutions*

Summary of forty-seven analyses (in duplicate or triplicate) of thirty-two "serum salt" solutions.

No. of analyses	Salt solution containing 5 m. eq. of Ca per liter		Average deviation of photoelectric from known values	Extremes of deviations of photoelectric from known values
	Volume of sample	Calcium content of sample		
	cc.	γ	per cent	per cent
6	1.0	100	±1.4	-2.4, +2.4
1	0.5	50	±2.2	-2.2, +2.2
16	0.2	20	±1.9	-4.0, +2.8*
7	0.1	10	±1.9	-3.2, +3.0
9	0.05	5	±1.1	-3.0, +1.4
8	0.02	2	±1.4	-2.2, +1.0†

\* One analysis, included in the average calculation, was 5 per cent too low.

† One analysis, not included in the average, was 6 per cent too high.

TABLE IV

*Results of Photoelectric Calcium Analyses (in Duplicate or Triplicate) of 84 Different Samples of Human Serum, Compared with Gasometric (Van Slyke and Sendroy (16)) Analyses*

No. of analyses	Serum sample		Average deviation of photoelectric from gasometric values	Extremes of deviations of photoelectric from gasometric values
	Volume	Approximate calcium content of sample		
	cc	γ	per cent	per cent
23	1.0	100	±0.8	-1.0, +1.8
1	0.5	50	0.0	0.0
21	0.2	20	±2.0	-3.5, +3.8
9	0.1	10	±1.8	-0.5, +3.4
19	0.05	5	±1.6	-4.1, +2.9*
11	0.02†	2	±1.9	-3.3, +3.5†

\* One analysis, not included in the average, was 7.3 per cent too high.

† Results for these samples by yellow color readings only.

‡ One analysis, included in the average, was 5.2 per cent too low.

ments.<sup>12</sup> Samples of 1.0 to 0.02 cc. were analyzed by the photoelectric method. For reference values, calcium was directly precipitated from triplicate 1.0 cc. samples, and analyzed by a slight modification (Sendroy,

<sup>12</sup> We are indebted to the physicians and personnel of the Mercy Free Dispensary, for providing these blood samples.

unpublished results) of the gasometric oxalate method (15), with an accuracy of  $\pm 1$  per cent.

The results, *which include the errors of both methods*, are given in Table IV. Agreement between photoelectric and reference values is almost as good for serum as for known calcium solutions. Deviations throughout all groups were within  $\pm 2.0$  per cent, with extremes of about  $\pm 4.0$  per cent.

### *Factors Affecting Results and Governing the Technique*

The many experiments done in a study of the essential factors involved at different stages in the development of the technique are briefly outlined.

*Effect of Water, Alcohol, and Ether Washing Mixtures*—To test the possibility of reduction of  $\text{Ce}(\text{SO}_4)_2$  by traces of residual organic substances from the W. A. E. mixture, two sets of dry and empty centrifuge tubes were prepared: one set was alternately centrifuged and washed with ammonia water and W. A. E. mixture, then dried; the other was not washed. To all of the tubes there were then added 0.5 cc. of 0.0005 N  $\text{Ce}(\text{SO}_4)_2$ ,  $\text{H}_2\text{SO}_4$ , KI, and alcohol, and readings were made under the conditions for 0.02 cc. analysis (Table I). With the reagents recommended for the W. A. E. mixture, and proper drying, reduction has rarely been observed, in many such comparisons. Reagents of different grade, especially ordinary 95 per cent alcohol, gave definite, but variable, reducing effects, and are therefore not to be used. A slight reducing effect, owing to traces of  $(\text{NH}_4)_2\text{C}_2\text{O}_4$  not removed in washing, though small, and not always observable, is controlled in the present method by the use of *standards* for each series of analyses.

It is obvious, from analyses of known calcium solutions (Table III), that *with the washing technique carried out as prescribed* no  $\text{CaC}_2\text{O}_4$  is dissolved in the wash fluid. This holds even for samples containing only 0.002 mg. of Ca, an amount which, when precipitated as oxalate, is just barely visible to the naked eye.

*Stability of Dilute Ceric Sulfate Solutions*—0.005 N, 0.002 N, 0.001 N, and 0.00035 N  $\text{Ce}(\text{SO}_4)_2$  solutions, prepared as prescribed, were mixed with appropriate amounts of  $\text{H}_2\text{SO}_4$ , KI, and alcohol when needed, to give readings of about 30 with filters No. 400 and No. 586-5. Fresh mixtures were made at hourly intervals, and read. With maintenance of initial readings within 0.1 considered a sign of stability, several experiments indicated that all of the above solutions were stable for 3 to 4 hours, then lost strength. Some solutions, not always the same ones in each experiment, were stable for 16 hours or longer. Exposure to sunlight accelerated deterioration. The stability of the  $\text{Ce}(\text{SO}_4)_2$  has no bearing on results when analyses of unknowns and *standards* are carried out simultaneously with the same oxidizing solution (p. 246).

*Effect of Reagents on Color Readings*—The composition of solutions in

which iodine is determined in this method differs from that of systems represented by curves previously used (p. 249). Curves like Curves C, E, and I, for known iodate solutions containing appropriate concentrations of  $\text{H}_2\text{SO}_4$ , KI, and  $\text{Ce}_2(\text{SO}_4)_3$ , with and without alcohol, were therefore studied, under the conditions outlined in Table I.

The readings and curves, with suitable amounts of  $\text{H}_2\text{SO}_4$  in place of  $\text{H}_3\text{PO}_4$  ("Calibrations") were exactly the same as those (Curves C, E, and I) of Sendroy and Alving (12) for the same iodine concentrations. When the samples stood in stoppered tubes for 1 or 2 hours, the readings remained constant. Against pure water tubes in place of reagent blanks, however, all readings were not only initially lower, but they decreased on standing. The indicated hydrolysis of KI with iodine formation is obviously adequately controlled by the use of reagent blanks. Nevertheless, the determination of calibration curves as outlined above is preferred, because hydrolysis is negligible under such conditions.

All iodine measurements in this method are made in the presence of  $\text{Ce}_2(\text{SO}_4)_3$ , the product of complete reduction of  $\text{Ce}(\text{SO}_4)_2$  by successive reaction with oxalate and iodide (Equations 1 and 2). The effect on readings of  $\text{Ce}_2(\text{SO}_4)_3$ , for which there is no control in the reagent blanks, was also studied, and found to be nil. Thus, proof was complete that yellow color readings obtained in the present method agree exactly with those of the calibration curves at the same iodine concentrations.

The conditions under which measurements of blue color, likewise sensitive to changes in acidity and composition of solutions, are made provide adequate control of the above factors. The readings obey Beer's law within the useful range (5, 9), and a new calibration line is always obtained with the standards for each group of measurements.

*Effect of Serum on Photoelectric Readings*—Analyses of 0.02 cc. samples of serum did not, at first, show the agreement indicated in Table IV, although results for corresponding samples of known salt solutions, containing 2  $\gamma$  of calcium, were satisfactory. The effect on the readings, of traces of residual serum present in the unknown sample tubes, and not present in the standards, was followed in many experiments embracing all of the procedures outlined in Table I. In addition to the tubes for serum samples and standards, there were also prepared two serum blanks in centrifuge tubes containing 1 volume of serum + 6 volumes of 0.12 N NaCl to replace  $(\text{NH}_4)_2\text{C}_2\text{O}_4$  and water. After standing overnight, all of the tubes, and in addition two other empty ones, were washed and dried as usual. Standard oxalate was added to the serum blanks as for the standards, also to the two other tubes known as standard controls. The rest of the procedure was carried out as usual, and readings made of yellow or blue colors. The difference in readings between the serum blank and

*standard control* was taken as the serum effect, and applied to readings of the unknown serum samples.

A study of 69 human serum samples showed an average change in yellow color readings of  $+0^2$  and  $+1^1$  at readings of 20 to 50 respectively, for 0.02 cc. analyses of fresh serum only. For serum more than 24 hours old, and for all analyses of 0.05 cc. or larger samples, fresh or not, corresponding effects averaging  $+0^0$  and  $+0^1$  were obtained. Since the correction is hardly significant otherwise, it is used only for the analysis of 0.02 cc. samples of fresh serum.

Presumably, the effect is a reduction of  $\text{Ce}(\text{SO}_4)_2$  by the serum proteins. On standing in the cold for more than 24 hours, additional protein (fibrin?) may separate out of serum taken from spontaneously clotted blood. This may explain the vanishing effect for 0.02 cc. analyses of stale serum. The non-effect in larger samples may be explained by the decreasing effect of a constant amount of residual serum in the presence of increasingly greater amounts of  $\text{Ce}(\text{SO}_4)_2$  used in larger samples.

A study of the serum effect on *blue color* readings showed no change in readings for serum samples of 0.05 cc. or larger. The increase in readings for 0.02 cc. samples, both fresh and stale, was so variable that *blue color readings for such samples (0.02 cc.) are not recommended as practicable or reliable, under any circumstances.*

*Further Remarks on Conditions of Analysis*—The particular concentrations of reagents used for procedures given in Table I were dictated by the requirements of the reactions involved, and were the subject of other studies too numerous for mention in detail.

The concentrations of  $\text{Ce}(\text{SO}_4)_2$  and standard oxalates were so designed as to give a range of iodine readings corresponding to  $\pm 50$  per cent ( $\pm 100$  per cent, only for 0.02 cc. analyses) of normal calcium values, within the optimum range of sensitivity and reliability of the photoelectric readings, *i.e.* between 20 and 50, with normal readings around 30. Thus, the adaptability of the method to the analysis of pathological sera is such as to embrace most of the values for calcium likely to be found in clinical work, between 2.5 and 7.5 milliequivalents per liter (between 5 and 15 mg. per 100 cc.).

It was found theoretically and experimentally more advantageous to use standard oxalates at the two ends of the useful range of color, rather than to standardize the  $\text{Ce}(\text{SO}_4)_2$  itself. Use of the latter alone, in amounts less than that added for reaction with oxalate, involves a multiplication of the analytical error, which, in the calculation of oxalate by differences, is transferred to the latter values. Standardization with amounts of  $\text{Ce}(\text{SO}_4)_2$  actually used for reaction usually involves readings which are too low for the best results.

Although the conditions described apply particularly to color readings with the Evelyn instrument, little or no modification should be required for measurements with other types of photoelectric colorimeters. Except for possible modifications necessitated by differences in the properties of other photoelectric cells, all that is needed is the possibility of making readings within the selected range of wave-lengths of light transmitted by the filters used in this work.<sup>9</sup>

The procedures given are also adaptable, to a limited extent, to visual colorimetry. Serum samples of not less than 0.2 cc. may be used, with blue color readings which are subject to factors the influence of which has been described, and which must be controlled (8).

The author is indebted to Edward J. Fitzsimons for his assistance in the analytical work of these studies.

#### SUMMARY

A method is described whereby calcium is directly precipitated as the oxalate, and measured by the extent of its reduction of a constant amount of ceric sulfate. The sensitive photoelectric technique of iodometry used for this purpose permits accurate analysis, to within an average error of  $\pm 2$  per cent, of as little as 0.002 mg. of calcium, or 0.02 cc. of serum.

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# THE DENATURATION OF PROTEINS AND ITS APPARENT REVERSAL

## IV. ENZYMATIC HYDROLYSIS OF NATIVE, DENATURED, AND APPARENTLY REVERSIBLY DENATURED PROTEINS

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In preceding papers of this series (1, 2) evidence was presented to show that the denaturation of horse serum albumin and pseudoglobulin by urea and guanidine hydrochloride was essentially an irreversible process in spite of the fact that the "regenerated"<sup>1</sup> proteins simulated in certain respects the properties of the native materials from which they were derived. With serum albumin, differences were observed in conditions and yield of crystallization, and with both albumin and globulin, differences in electrophoretic mobility (3).<sup>2</sup>

While these findings can be regarded as conclusive proof for the irreversible nature of the denaturation process under the conditions described, they do not demonstrate whether the observed differences between native and regenerated proteins are due to differences in details of the surface properties of the molecules or whether they reflect more fundamental differences in their intrinsic structures.

It has been shown that enzymatic hydrolysis of certain proteins proceeds more readily when the protein is in the denatured state. From measurements of the rates of tryptic digestion of native and denatured egg albumin, Lin, Wu, and Chen (4) concluded<sup>3</sup> that "since the rate of digestion of

<sup>1</sup> The term "regenerated" protein denotes the fraction which in the preceding papers of this series (1, 2) was referred to as "reversibly" denatured protein. It is used, for lack of a better term, to indicate that part of the denatured material which approximates most closely to the physicochemical properties of the native protein.

<sup>2</sup> Sharp, D. G., Cooper, G. R., Erickson, J. O., and Neurath, H., *J. Biol. Chem.*, **144**, 139 (1942).

<sup>3</sup> The data as given in their original paper do not substantiate this conclusion in that alkali-heated, acid-heated, alcohol-denatured, and acid-denatured egg albumin appear to exhibit a lower rate of tryptic digestion than the native protein. In view of the conclusions drawn in the discussion and summary of that paper, it may be possible that in the legends to Fig. 4 and headings of Tables 7 and 8 "native" and "alkali-heated" egg albumin have been accidentally interchanged, in which case the data would be in agreement with the text.

natural albumin by trypsin is exceeded by that of all denatured albumins, including those by alcohol and shaking, it is probable that the fundamental process of denaturation is a sort of incipient tryptic digestion." Observations of differences in the temperature coefficient of the tryptic digestion of native and heat-treated lactoglobulin led Linderström-Lang (5, 6) to the belief that denaturation is a prerequisite for the tryptic digestion of native globular proteins, a hypothesis which appears to receive support from physicochemical studies on the action of papain on thyroglobulin (7). While this may be open to criticism, the fact seems to be established that the denatured protein is digested more readily than the native. Hence, by comparing the rates of tryptic digestion of native, denatured, and regenerated protein it should be possible to decide whether the regenerated protein approximates more closely to the structural properties of the native or to those of the denatured material. The results of such an investigation on the so called reversible denaturation of horse serum proteins by urea are presented in this paper.

### *Materials and Methods*

Crystalline horse serum albumin, Fraction A, and horse serum pseudoglobulin GI were prepared as described in the preceding papers. Denaturation was effected by 8 M urea solutions and the irreversibly denatured and regenerated materials were recovered as described (1, 3). This involved removal of the urea by exhaustive dialysis, isoelectric precipitation of the irreversibly denatured proteins, and fractional precipitation of the water-soluble, regenerated proteins with sodium sulfate and ammonium sulfate, respectively.

Pancreatin u.s.p. X (Merek) was used as a source of tryptic activity. The progress of proteolysis was measured by formol titrations of aliquots of solutions containing known amounts of enzyme and substrate which were incubated in a water bath at  $37^{\circ} \pm 0.1^{\circ}$ . The substrate was dissolved in 3 cc. of a 0.05 M phosphate buffer of pH 7.8 and 1 cc. of buffer solution containing the enzyme was added. 0.1 cc. of toluene was added, the solutions were incubated, and at specified time intervals 0.5 cc. samples were removed, diluted with 5 cc. of distilled water, and titrated with a standard 0.01 N sodium hydroxide solution to the end-point, with phenolphthalein as indicator. After addition of 1 cc. of a 40 per cent neutralized formaldehyde solution, the solutions were titrated as before. Blank determinations on solutions containing pancreatin and substrate respectively were carried out with each series of measurements and the correction for the mild autolysis of the pancreatin applied to the data. There was no measurable autolysis of the substrates. The data presented here are the results of duplicate determinations.

## Results

The influence of enzyme concentration on the rate of hydrolysis of native and regenerated serum albumin was determined by adding 10, 25, and 40 mg. of pancreatin to solutions containing 50 mg. of substrate. With both the native and the regenerated protein, the rates increased with increasing enzyme concentration, approaching a maximum when the enzyme concentration was about 40 mg. per 50 mg. of substrate. As may be seen from Fig. 1, in which the time of incubation is plotted against the amount of 0.01 *N* NaOH required to neutralize 10 mg. of substrate, the rate of hydrolysis of the regenerated protein exceeded that of the native protein in each series of measurements.

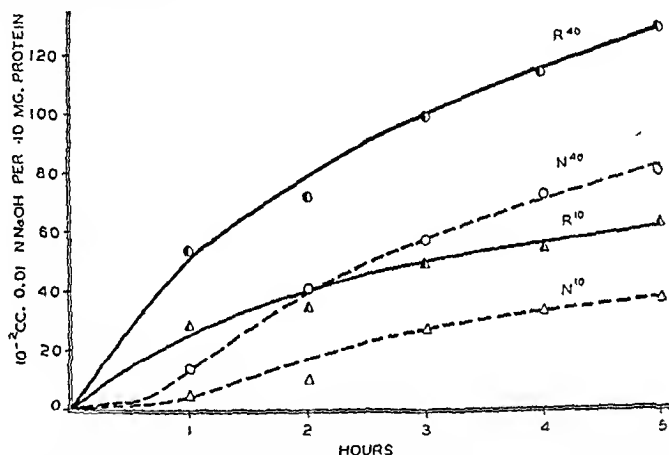


FIG. 1. Tryptic hydrolysis of native and regenerated serum albumin. The lower two curves refer to the hydrolysis of 50 mg. of native and regenerated serum albumin by 10 mg. of pancreatin ( $N^{10}$ ,  $R^{10}$ ), the upper two curves to the hydrolysis by 40 mg. of pancreatin ( $N^{40}$ ,  $R^{40}$ ).

In Fig. 2 the rates of hydrolysis of 50 mg. each of native, denatured, and regenerated albumin by 40 mg. of pancreatin are compared with one another. It will be noted that the regenerated and denatured materials are both hydrolyzed at the same rate, whereas the hydrolysis of the native material proceeds considerably more slowly.

In Fig. 3 the analogous data are given for native, irreversibly denatured, and regenerated pseudoglobulin GI. In these measurements 0.1 *N* NaCl was added in order to render the irreversibly denatured material more soluble.

The substrate concentration was 50 mg., the enzyme concentration 40



mg. in 4.5 cc. of solution. The rates of hydrolysis were considerably lower than those observed for the albumin fractions; hydrolysis appeared to come to a standstill after about 4 hours. The rate was lowest for native pseudo-

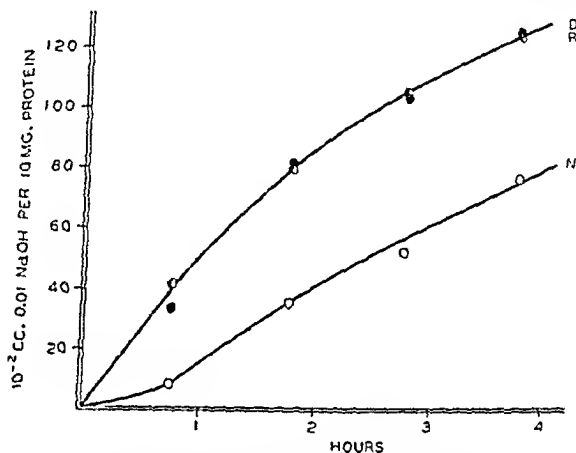


FIG. 2. Tryptic hydrolysis of 50 mg. of native (O), irreversibly denatured (●), and regenerated (●) serum albumin by 40 mg. of pancreatin.

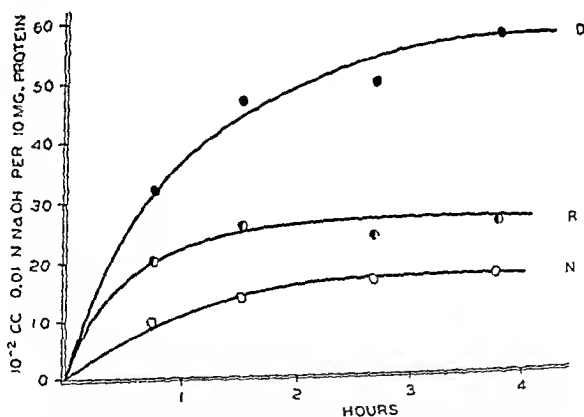


FIG. 3. Tryptic hydrolysis of 50 mg. of native (O), irreversibly denatured (●), and regenerated (●) pseudoglobulin GI by 40 mg. of pancreatin.

globulin, highest for the irreversibly denatured fraction, and intermediate for the regenerated fraction. The same relative order was observed in measurements on solutions containing 27 mg. of substrate per 40 mg. of enzyme.

## DISCUSSION

The present data demonstrate conclusively that the *rates* of hydrolysis of the native albumin and pseudoglobulin are considerably lower than those of the respective irreversibly denatured materials. They also show that hydrolysis proceeds to a greater *extent* when the proteins are in the denatured state than when they are in the native configuration. These differences can hardly be ascribed to variations in the optimal pH regions, for the data of Lin, Wu, and Chen (4) clearly indicate that the rates of proteolysis of the native and denatured proteins, although to some extent dependent on pH, never overlap within the pH stability range. Preliminary measurements did not reveal any measurable variation of the rates of hydrolysis with pH, within the range of pH 7 to 9.

The data shown in Fig. 2 leave no doubt that, with respect to the ease of proteolytic fission, the regenerated albumin is actually in the denatured state. This finding is in full accord with the observations and ideas recorded in the preceding papers (1, 2).

With pseudoglobulin, the response to enzymatic attack is similar to that observed for the albumin in that the irreversibly denatured fraction is hydrolyzed more readily than the native protein. However, the rate of digestion of the regenerated fraction is much lower than that of the denatured fraction, and only somewhat higher than that of the native. These differences in behavior of albumin and globulin become of greater significance when considered together with those observed in the preceding studies (1-3).

While the irreversibly denatured and the regenerated albumin are distinguished from the native protein by a lack of a specific intrinsic configuration, the configuration of the regenerated globulin may be thought of as being only slightly more random than that of the native globulin. Native and regenerated globulin may conceivably be equally susceptible to enzymatic attack and the relatively small differences observed experimentally appear to sustain this idea. The greater susceptibility of the irreversibly denatured globulin could be accounted for by its more extended configuration (3) which renders it more accessible to the enzyme.

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## SUMMARY

In an attempt to detect differences in the internal structure of native, of irreversibly denatured, and of so called regenerated proteins, measurements of the rates of tryptic hydrolysis of such preparations derived from horse

serum albumin and horse pseudoglobulin GI have been carried out. With both proteins, proteolysis proceeds more slowly with the native than with the irreversibly denatured material. The extent of proteolysis is larger with the albumin than with the globulin fractions.

The proteolytic rates of the irreversibly denatured and of the regenerated serum albumin are equivalent, indicating that the regenerated protein is indeed essentially in a denatured state, and that the denaturation under the condition described was actually an irreversible process. With pseudoglobulin GI the rate of tryptic digestion of the regenerated fraction was lower than that of the irreversibly denatured fraction and only somewhat higher than that of the native protein. The findings are discussed in terms of structural differences between serum albumin and pseudoglobulin.

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# MECHANISM OF BIOLOGICAL NITROGEN FIXATION

## IX. PROPERTIES OF HYDROGENASE IN *AZOTOBACTER*\*

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Recently Phelps and Wilson (1) reported the occurrence of hydrogenase, the enzyme which activates molecular hydrogen, in nitrogen-fixing bacteria. Because  $H_2$  specifically inhibits nitrogen fixation by red clover plants inoculated with *Rhizobium trifolii* as well as by *Azotobacter* (2, 3), possession of hydrogenase by these organisms may be more than merely fortuitous. The possibility must be considered that hydrogenase is associated with nitrogenase, the enzyme concerned in the first step of the fixation reaction. Evidence which supports the view of a relationship was their observation that cultures of the root nodule bacteria taken directly from nodules of the pea plant fixing nitrogen contained hydrogenase but not those grown on laboratory media (on which they are unable to fix  $N_2$ ). This significant finding, however, could not always be confirmed with cultures from the nodules, apparently because the factors which affect hydrogenase activity in nitrogen-fixing bacteria were not known. In the preliminary investigations we followed the technique described by Stephenson and her associates (4) for studies of hydrogenase in *Escherichia coli* and related species, but this may not be satisfactory, since the metabolism of the nitrogen-fixing bacteria is quite different from that of the colon group. We have therefore determined the chief properties of hydrogenase as it occurs in *Azotobacter* in order to develop a method which will insure consistent results in the further study of its possible relationship to nitrogen fixation.

### Methods

All experiments were made with a culture of *Azotobacter vinelandii* which under optimum conditions fixes 15 to 20 mg. of N per 100 ml. in 24 hours (5). Resting cell suspensions in Allison's solution were prepared by the method of Wilson (6) from 36 hour cultures grown in Roux bottles on Burk's nitrogen-free medium plus 2 per cent agar. Gas exchanges were measured by the usual Warburg micro respirometer technique. Unless otherwise stated, 1 ml. of cell suspension and 2 ml. of phosphate buffer (pH 7.5) were placed in each flask; the temperature of the bath was 34-35°.

\* This research was supported in part by grants from the Rockefeller Foundation and from the Wisconsin Alumni Research Foundation.

## Concentration of Cells

When  $H_2$  from which all trace of  $O_2$  had been removed was supplied to Warburg flasks containing *Azotobacter* suspensions, little or no uptake of gas occurred unless suitable acceptors (fumarate, nitrate, methylene blue) were added. When methylene blue was used, uptake of  $H_2$  quantitatively equal to that necessary for complete reduction of dye was noted.<sup>1</sup> Following the suggestion of Phelps and Wilson (1) that molecular oxygen may serve as a hydrogen acceptor in this system, cell suspensions were supplied with a gas mixture of 80 per cent  $H_2$  and 20 per cent  $O_2$ . For controls the hydrogen was replaced with helium, argon, or nitrogen (air). In a typical experiment the  $Q_K(N)$  in the  $H_2$ - $O_2$  system was 3500 as compared with a

TABLE I

*Effect of Cell Wash Solution and Glucose on  $Q_K(N)$  and  $Q_{O_2}(N)$*

$Q_K(N)$  is based on the uptake of gas in an atmosphere of  $pO_2$  0.2 and  $pH_2$  0.8 atmosphere;  $Q_{O_2}(N)$  on the uptake of gas in an atmosphere of  $pO_2$  0.2 and  $pHe$  0.8 atmosphere (KOH in center well).

Experiment No.	Concentration of cells	Addition	$Q_K(N)$	$Q_{O_2}(N)$
	mg. N per ml.			
1	0.56	None	2500	118
		Wash*	2150	1270
	0.056	None	430	100
		Wash	3550	1250
2	0.48	None	1870	120
		Glucose†	2090	350
	0.16	None	1000	100
		Glucose	2160	450

\* Wash, 1 ml. of Allison's solution used for the first washing of the cells.

† Glucose, 0.001 M.

$Q_{O_2}(N)$  of 80 in the argon control and 100 in the air control.<sup>2</sup> These data furnish strong evidence that  $H_2$  as well as  $O_2$  is being consumed by the cells.

In the foregoing experiment a very heavy suspension of bacteria was used; so that the actual uptake of gas in the  $H_2$ - $O_2$  atmosphere was about 1000 c.mm. per hour. Such a large uptake cannot be accurately controlled because of manipulative and other technical difficulties; e.g., diffusion effects. Dilution of the suspension caused a sharp drop in the  $Q_K(N)$

<sup>1</sup> The high "endogenous" uptake of gas reported by Phelps and Wilson (1) likely arose from impurities of  $O_2$  remaining in the  $H_2$  used.

<sup>2</sup>  $Q_K(N)$  = c.mm. of total gas uptake per hour per mg. of N in the cells; K refers to the fact that this oxidation of  $H_2$  is frequently called the Knallgas reaction.  $Q_{O_2}(N)$  = c.mm. of  $O_2$  uptake per hour per mg. of N in the cells.

value. For example, in the experiment cited in Table I, a concentrated suspension of *Azotobacter* (0.56 mg. of N per ml.) possesses a  $Q_K(N)$  value of 2500; when the cells were diluted 1:10, it decreased to 430. This effect of dilution, which is frequently encountered in enzyme studies, usually signifies that some necessary soluble component of the system has been more or less completely removed by the washing procedure. Addition of the Allison's solution used to wash the cells in preparing the suspension completely restored the hydrogenase activity.

Further investigation showed that the stimulating factor in the cell wash solution was heat-stable and apparently could be replaced by a suitable substrate such as glucose or sucrose (Table I). Although the addition of either cell wash solution or substrate overcomes the technical difficulty of decrease (or even disappearance) of  $H_2$  uptake with decrease in concentration of the cells, the method has the disadvantage that it causes the  $Q_O$  to become comparable in magnitude with the  $Q_K$ . For many purposes this is undesirable. A number of other soluble factors including riboflavin and cozymase which might have been lost in the washing were tested for ability to restore the hydrogenase activity in dilute suspensions, but none was effective. It is evident from these experiments that the concentration of washed cells used is an important factor in determining the activity of hydrogenase in cells of nitrogen-fixing bacteria and that this must be carefully controlled in a study of the characteristics of the enzyme.

### *pO<sub>2</sub> Function*

A possible explanation of the effect of cell concentration was furnished by investigations which determined the influence of the  $pO_2$  on the system. In these experiments, the  $pH_2$  was maintained at 0.4 atmosphere, the remaining gas being He or  $N_2$ . As is evident from the data in Fig. 1, the optimum  $pO_2$  varied with the concentration of cell suspension: the heavier the suspension, the higher the optimum. With heavy suspensions a  $pO_2$  such as was used in the preceding experiments is satisfactory, but with more dilute ones such a  $pO_2$  is much too high. These results suggest that a low  $pO_2$  is necessary with low cell concentration; otherwise the enzyme system is inactivated by oxidation. With a high  $pO_2$  substrate must be present to keep the enzyme reduced; this can be accomplished either by employing a heavy cell suspension or adding substrate (wash water or carbohydrate). Inhibition of hydrogenase by oxygen has been noted in numerous bacteria as well as algae (7-10).

Since it is not feasible to use very heavy suspensions because of too rapid uptake of gas, in subsequent work the cell suspension was standardized by means of turbidity measurements with a Coleman photoelectric colorimeter, so that it contained approximately 0.1 mg. of N per ml. Each

Warburg flask received 1 ml. of such a suspension and was supplied with a gas mixture in which the  $pO_2$  was 0.025 to 0.05 atmosphere. The data obtained with two typical suspensions show that under these conditions the maximum  $Q_K$  is likely obtained (Table II).

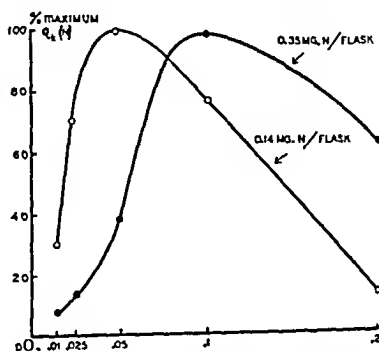


FIG. 1. Dependence on the  $pO_2$  of hydrogenase activity in different concentrations of *Azotobacter* cells.  $pH_2$  0.40 atmosphere,  $N_2$  as diluent,  $pH$  7.5,  $35^\circ$ .

TABLE II

$Q_K(N)$  with Different  $pO_2$  in Gas Mixture

Each flask contained 0.115 mg. of N in both experiments;  $pH_2 = 0.4$  atmosphere, helium to 1.0 atmosphere.

Experiment No.	$pO_2$ in atmosphere				
	0.01	0.025	0.05	0.10	0.20
1	1000	2460	2085	900	363
2	1435	1610	1270	557	191

### $pH_2$ Function

Studies on the  $pH_2$  function summarized in Fig. 2 indicate that this is a typical activity-substrate curve. Differences in the rate of uptake of gas cannot be detected in the  $pH_2$  range from 0.1 atmosphere to 0.95 atmosphere; below the lower limit the activity drops sharply. Because the substrate is in the gas phase, calculation of a Michaelis constant from these data may be subject to error, since diffusion of gas to the enzyme is probably a factor. Support for this view is furnished by the data of Fig. 3. With a dilute suspension of cells a  $K_m$  value of 0.025 atmosphere is indicated; with a heavier suspension, one of 0.04 atmosphere. For practical purposes, however, it appears that if a  $pH_2$  of at least 0.4 atmosphere is used the enzyme will be saturated with substrate.

pH function resembles more closely those for assimilation of different forms of combined nitrogen by *Azotobacter* (11).

The response of the hydrogenase system to temperature is illustrated in Fig. 5. The data are from two separate experiments, but the agreement is so close that it is unnecessary to adjust the observed values before they are combined. The optimum temperature is about 40°, and the  $\mu$  value (6) based on the fourteen observations from 21–40° is  $11,200 \pm 670$  calories. The corresponding constants for nitrogen fixation are 33° and 19,300 calories and for assimilation of  $\text{NH}_3\text{-N}$ , 37° and 19,300 calories. It should be noted, however, that the  $\mu$  values for assimilation of free and combined

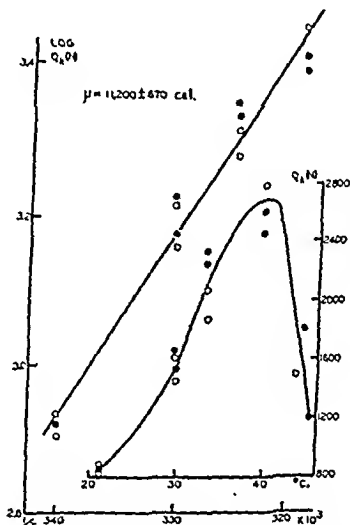


FIG. 5. The temperature characteristics of hydrogenase in *Azotobacter*

nitrogen (which are also identical with that for respiration by *Azotobacter*) were obtained with growing cultures and not with a "resting" suspension. Burk (11), who reports these values, is of the opinion that they are associated with activation of the carbohydrate component of growth rather than with the nitrogen assimilation process.

#### SUMMARY

The chief properties of *hydrogenase* in *Azotobacter* cells have been determined with molecular oxygen as the hydrogen acceptor. Both the  $p\text{O}_2$  and  $p\text{H}_2$  functions vary with the concentration of cells. The optimum pH is about 7.5 and the optimum temperature, 40°. Consideration of these



# MECHANISM OF BIOLOGICAL NITROGEN FIXATION

## X. HYDROGENASE IN CELL-FREE EXTRACTS AND INTACT CELLS OF AZOTOBACTER\*

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(From the Department of Agricultural Bacteriology, University of Wisconsin, Madison)

(Received for publication, March 23, 1942)

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### Methods

The methods for measuring hydrogenase activity in resting suspensions of *Azotobacter* have been described in previous papers (1, 2). For the cell-free extract *Azotobacter vinelandii* was grown in nine liter serum bottles or in a 200 gallon fermenter (3); 24 to 36 hour cultures were concentrated to a paste by passing through a Sharples supercentrifuge (45,000 R.P.M.). To make a cell-free enzyme preparation, we have used a method developed by Wiggert *et al.* (4). The cell paste is washed in M/15 phosphate buffer (pH 7.0); then 1 part of paste is mixed with 2 parts of powdered glass. More buffer is added until the cell-glass mixture has the consistency of a thick batter. This mixture is ground in an all-glass bacterial mill designed by Werkman and his collaborators.<sup>1</sup> The cell debris, glass, and uninjured cells are re-

\* This research was supported in part by grants from the Rockefeller Foundation and from the Wisconsin Alumni Research Foundation.

<sup>1</sup> We express our appreciation to Professor Werkman and his associates for demonstrating to us in their laboratory many of the techniques which they have developed for preparing cell-free enzymes from bacteria but which are not yet published. Our thanks are also due Professor J. W. Williams of the Department of Chemistry, University of Wisconsin, for his aid in the use of the Beams ultracentrifuge in that department.



# MECHANISM OF BIOLOGICAL NITROGEN FIXATION

## X. HYDROGENASE IN CELL-FREE EXTRACTS AND INTACT CELLS OF *AZOTOBACTER*\*

By S. B. LEE, J. B. WILSON, AND P. W. WILSON

(From the Department of Agricultural Bacteriology, University of Wisconsin, Madison)

(Received for publication, March 23, 1942)

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moved from the juice by centrifuging at 4500 R.P.M. for 45 minutes. Further clarification is obtained by centrifuging in a Beams ultracentrifuge at 24,000 R.P.M. for 30 minutes, followed by aseptic filtration through a Berkefeld N or a Mandler No. 15 filter. Such a preparation showed no growth when used to inoculate a nitrogen-free medium.

### *Hydrogenase in Cell-Free Preparations*

The original studies on hydrogenase made by Stephenson and her associates indicated that this enzyme was rather fragile in resting suspensions of the colon group of organisms. It might be thought then that cell-free preparations would be difficult to prepare, but at least two groups of workers (5, 6) have been successful. Hydrogenase in suspensions of *Azoto-*

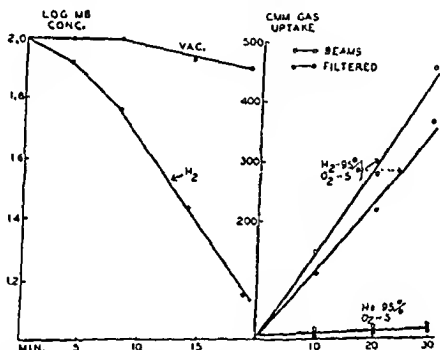


FIG. 1. Hydrogenase in a cell-free enzyme preparation from *Azotobacter*. Left, reduction of methylene blue in a modified Thunberg tube; right, uptake of gas in presence and absence of hydrogen. "Beams" refers to supernatant liquid from centrifugation in the Beams centrifuge; "filtered," same material after filtration. "Vac." means vacuum control.

*bacter* is quite stable; we have kept a suspension in the refrigerator at 3° for 6 weeks with little loss in activity. Moreover, both acetone preparations made according to Bovarnick (6) as well as cell extracts contain a highly active hydrogenase. The acetone preparations are not so satisfactory as the cell-free extracts; so we have not studied the properties of the former to a great extent. As is shown in Fig. 1, these cell-free preparations of *Azotobacter* not only decolorize methylene blue in the presence of hydrogen but also take up gas in an  $H_2-O_2$  mixture much more rapidly than in an  $He-O_2$  mixture, indicating the oxidation of hydrogen. So far as we know this is the first demonstration of the Knallgas reaction brought about by a cell-free enzyme preparation.

### Mechanism of Hydrogen Utilization

That the cell-free enzyme preparation is very similar to the enzyme in the intact cell was demonstrated in a series of experiments in which the mechanism of hydrogen utilization by *Azotobacter* was investigated. Previous studies in which it was shown that more gas disappeared in an  $H_2-O_2$  mixture than in air or an  $He-O_2$  mixture provide indirect evidence that hydrogen was being oxidized. More direct evidence which demonstrates that hydrogen actually disappears is furnished by the data in Figs. 2 and 3. Suspensions of *Azotobacter* cells or cell-free preparations were furnished atmospheres containing limited quantities of oxygen. As can be seen in Figs. 2 and 3, the total gas uptake in the presence of  $H_2$  greatly exceeds the

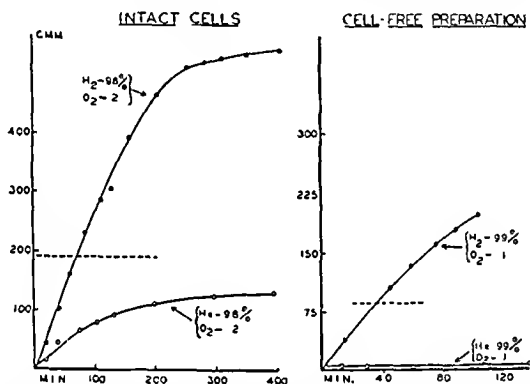


FIG. 2. Uptake of gas in presence and absence of hydrogen by resting suspensions and cell-free extracts of *Azotobacter*. The dotted lines indicate the theoretical oxygen uptake based on the oxygen content of the  $H_2-O_2$  mixture.

total quantity of  $O_2$  present (estimated through absorption by alkaline pyrogallol in a duplicate flask). Evidently molecular hydrogen must have disappeared. That its disappearance is due to combination with  $O_2$  is shown by the data in Fig. 3. When the rate of gas uptake in the  $H_2-O_2$  mixture approaches zero, undoubtedly because of exhaustion of  $O_2$ , addition of the latter causes immediate stimulation in the rate of uptake.

The quantitative aspects of the disappearance of gas are significant. In a number of experiments with both intact cells and cell-free preparations gas uptake in  $H_2-O_2$  was allowed to continue until no further decrease was evident. On the assumption that all the oxygen present combined with hydrogen 1.6 to 1.8 parts of  $H_2$  disappeared for each part of  $O_2$ . If, however, it is assumed that the same amount of  $O_2$  was used in respiration in the  $H_2-O_2$  mixture as was observed in the  $He-O_2$  control, the ratio becomes

3 to 4 parts of  $H_2$  for each part of  $O_2$ . It is probable that actually the respiration and hydrogen oxidation processes compete for the  $O_2$ ; so that the first assumption gives too low a value and the second, too high. The true ratio is very probably the theoretical 2:1 required for oxidation of hydrogen to water, and likely most of the  $H_2$  is utilized in this reaction rather than by union with other acceptors in the cell.

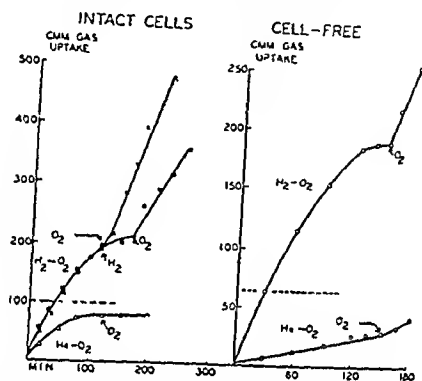


FIG. 3. Effect of addition of oxygen on gas uptake in presence and absence of hydrogen by intact cells and cell-free extracts of *Azotobacter*. Arrows denote the points at which the indicated gases were added. 1.0 per cent oxygen was used in gas mixtures with intact cells; 0.7 per cent in gas mixtures with the cell-free preparation. The dotted lines indicate the theoretical oxygen uptake based on the oxygen content of the  $H_2-O_2$  mixture.

### Effect of Inhibitors

Another striking similarity in the properties of the two types of preparations is in their response to the inhibitors which are usually associated with the activity of the cytochrome system in oxidation processes. Although cyanide stimulates hydrogenase activity when methylene blue is used as the acceptor (1), 0.001 M KCN almost completely inhibits oxidation of hydrogen by either cell suspension or cell-free enzyme preparation. At a pH of 6.5, 0.01 M sodium azide also inhibits although not nearly so effectively. Data from an experiment in which the suspension and extract were from the same batch of cells are given in Table I.

### Hydrogenase and Nitrogen Fixation in *Azotobacter*

The development of methods which allow consistent and reproducible estimation of hydrogenase activity in both intact cells and extracts of *Azotobacter* provides a means for determining the relationship, if any, be-

tween this enzyme and the nitrogen-fixing system in the organism. Two types of experiments which illustrate the method of attack will be briefly described.

*Source of Nitrogen*—To determine the effect of the source of nitrogen on the hydrogenase activity in *Azotobacter vinelandii*, we have transferred cultures of this organism every 24 to 36 hours for 10 days on the usual nitrogen-free agar medium and on the same medium plus 350 p.p.m. of  $\text{NH}_4\text{NO}_3\text{-N}$ . These two cultures were then used for inoculation of the

TABLE I

*Effect of Inhibitors on Hydrogenase from Azotobacter*

Tests with 0.001 M KCN at pH 7.5 and with 0.01 M sodium azide at pH 6.5.

Source of hydrogenase	Inhibitor	Gas uptake <i>c.mm. per hr.</i>	Inhibition <i>per cent</i>
Intact cells	None	385	
	KCN	0	100
	None	297	
	Azide	223	25
Enzyme preparation	None	350	
	KCN	26	92
	None	350	
	Azide	86	75

TABLE II

*Nitrogen Content of Azotobacter vinelandii*

Suspension from	Gas uptake <i>mg N per flask</i>	Methylene blue reduction <i>mg N per tube</i>
$\text{NH}_4\text{NO}_3$ culture grown on N-free medium	1.17	0.0234
N-free " " " " "	1.06	0.0212
$\text{NH}_4\text{NO}_3$ " " " $\text{NH}_4\text{NO}_3$ "	1.32	0.0220
N-free " " " " "	1.80	0.0257

same media in Roux bottles for preparing suspensions which were tested for hydrogenase activity. Table II gives the nitrogen content of these suspensions.

The data in Fig. 4 demonstrate that the hydrogenase content of *Azotobacter vinelandii* is definitely associated with its nitrogen nutrition. When growing in N-free medium and hence forced to use molecular nitrogen, the organism possesses an active hydrogenase. If, however, nitrogen fixation is inhibited by  $\text{NH}_4\text{NO}_3$ , a marked decrease in the hydrogenase of the cells is observed. These responses to the source of nitrogen are apparently independent of the previous nitrogen nutrition of the organism.

root nodule bacteria from nodules of peas fixing nitrogen but not in the same culture of bacteria kept on laboratory medium, on which they do not fix nitrogen (1). (3) As noted in this report, *Azotobacter* grown on  $\text{NH}_4\text{NO}_3$  contains little hydrogenase in comparison with cells of this organism using atmospheric nitrogen.

*Evidence Indicating Distinct Enzyme Systems*—(1) The pH, temperature, and probably other functions for fixation of nitrogen and for oxidation of hydrogen by *Azotobacter* cells are quite different (2). (2) Molecular nitrogen does not inhibit hydrogenase activity.

We believe that on the whole present available evidence favors the view of some relationship. The most significant observation in support is the close association of hydrogenase activity with functioning of nitrogen fixation. Furthermore, part of the negative evidence is not critical, since other enzyme systems show differences when supplied different substrates. Also, Burk (9) furnishes convincing argument that the temperature function, especially the large  $\mu$  value for nitrogen fixation by *Azotobacter*, reflects a growth process rather than assimilation of free  $\text{N}_2$ . In all probability, if the systems responsible for oxidation of hydrogen and for fixation of nitrogen are related, the relationship will be similar to that between the systems for assimilating free and combined nitrogen (9) in that the nitrogen-fixing system will be more complex and probably contain components unnecessary for the functioning of hydrogenase. The absence of the  $\text{OH}^-$  component in the hydrogenase system indicated by the differing pH function is an example.

The fact that molecular nitrogen fails to inhibit hydrogenase does appear significant, but differences in the experimental conditions may explain why  $\text{H}_2$  inhibits nitrogen fixation but  $\text{N}_2$  does not inhibit oxidation of hydrogen. The former has been established with growing cultures, the latter with resting cells and cell extracts. If, with these preparations, the limiting factor is decomposition of some intermediate compound rather than its formation, reduction in the velocity of its formation through the presence of  $\text{N}_2$  would not be evident from the measurements. The most critical points of the evidence are being investigated further, and until these more detailed investigations are complete, final decision must be reserved.

#### SUMMARY

A method is described for preparing a cell-free enzyme extract of *Azotobacter* which contains an active hydrogenase capable of transferring molecular hydrogen to methylene blue or molecular oxygen. Evidence is presented that the uptake of gas in an  $\text{H}_2\text{-O}_2$  mixture by either cell-free preparation or resting suspension consists primarily in the oxidation of



hydrogen to water. Both cyanide and azide inhibit oxidation of hydrogen by cell suspensions or by the cell-free preparations.

The hydrogenase activity of *Azotobacter* grown on a medium containing  $\text{NH}_4\text{NO}_3$  is markedly decreased in comparison with that of cells fixing nitrogen. Molecular nitrogen does not inhibit hydrogen oxidation by either cell suspensions or cell-free extracts. These observations together with others made in previous reports in this series are discussed from the point of view of a possible relationship between hydrogenase and biological nitrogen-fixing systems.

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THE ISOLATION OF STILBESTROL MONOGLYCURONIDE FROM  
THE URINE OF RABBITS

Sirs:

Stilbestrol (4,4'-dihydroxy- $\alpha,\beta$ -diethylstilbene) synthesized by Dodds *et al.*<sup>1</sup> differs from the naturally occurring estrogens in certain important respects. The ratio of oral to parenteral effectiveness is much higher, and it is excreted in the urine in much larger amounts after administration. Both phenomena suggest a less drastic degradation of the synthetic estrogen in the body than is undergone by the natural estrogens. It is generally held that the liver plays the major rôle in estrogen inactivation. The natural estrogens have been isolated from the urine in conjugated form, as sulfates and glucuronides, as well as in the free state.

The fate of stilbestrol has been studied by Zondek and Sulman,<sup>2</sup> and by Stroud.<sup>3</sup> The latter isolated from the urine of rabbits 25 per cent of the stilbestrol injected. Approximately half of this was recovered as free stilbestrol, the remainder after acid hydrolysis. The conjugated form was not isolated as such, or otherwise identified.

We wish to report, as part of a study of the intermediary metabolism of stilbestrol and related compounds, the isolation and identification of stilbestrol monoglycuronide from the urine of rabbits after the intramuscular injection of stilbestrol. Pooled urine was made acid to Congo red with hydrochloric acid, and subjected to continuous ether extraction. From the ether extract which contained both free and conjugated stilbestrol, the sparingly soluble sodium salt of stilbestrol glycuronide was extracted with a saturated solution of sodium bicarbonate. A second extraction with 10 per cent sodium hydroxide yielded the free stilbestrol. The sodium salt of the glycuronide was then converted to the free acid with hydrochloric acid.

The glycuronide crystallized in the form of needles from acetone-benzene. After drying *in vacuo* at 110°, a product was obtained with a melting point of 175° and  $[\alpha]_D^{20} = -56.6^\circ$  (1.6 per cent in ethyl alcohol). The equivalent weight, determined by titration with sodium hydroxide in 25 per cent alcohol, was 450; calculated for the monoglycuronide, 444; for the digly-

<sup>1</sup> Dodds, E. C., Golberg, L., Lawson, W., and Robinson, R., *Nature*, **141**, 247 (1938).

<sup>2</sup> Zondek, B., and Sulman, F., *Nature*, **144**, 596 (1939).

<sup>3</sup> Stroud, S. W., *J. Endocrinology*, **1**, 201 (1939).

curonide, 620. Elementary microanalyses:<sup>4</sup> found, C 64.5, H 6.5; calculated for  $C_{24}H_{28}O_8$ , C 64.8, H 6.4. The Benedict test was negative with the glucuronide, but became positive after acid hydrolysis, indicating a glycosidic linkage. Acid hydrolysis of the glucuronide yielded free stilbestrol which was isolated in crystalline form and had a melting point identical with the pure compound injected, 168°. Elementary analyses:<sup>4</sup> found, C 80.1, H 7.5; calculated for  $C_{18}H_{20}O_2$ , C 80.5, H 7.5. The filtrate from the hydrolysis gave a strongly positive naphthoresorcinol test for glucuronic acid.

Following the administration of 3.0 gm. of stilbestrol in divided doses over 14 days, 1.5 gm. of stilbestrol glucuronide were isolated from the urine, a recovery of 30 per cent of the administered stilbestrol in conjugated form. This figure is undoubtedly less than the total amount of glucuronide excreted, since it refers to isolated material only. Its magnitude indicates the significance of this conjugating mechanism for the intermediary metabolism of stilbestrol.

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<sup>4</sup> We are indebted to Mr. W. Sasehek and Professor H. T. Clarke of the College of Physicians and Surgeons, Columbia University, for the microanalyses reported. The stilbestrol used in these studies was kindly contributed by Dr. F. Schmelkes of the Wallace-Tiernan Laboratories, Belleville, New Jersey. This study was aided by the Wyeth Endocrine Fund.

# QUANTITATIVE ESTIMATION OF PENICILLIN

Sirs:

Various groups of workers on penicillin concur with the idea that at present an urgent requirement is a sensitive and reliable method for quantitative determination of this and other antibiotic material. Following the discovery that graded doses of penicillin produce quantitatively a proportional inhibition of growth of *Staphylococcus aureus* in nutrient broth, a simple, rapid, and relatively sensitive method of assay has been developed. Growth is measured turbidimetrically as a function of penicillin concentration.

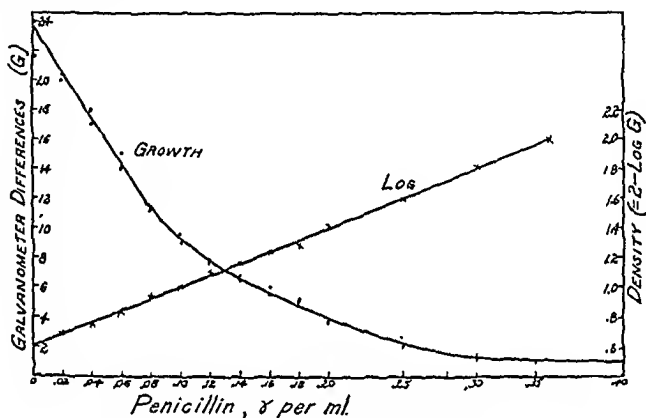


FIG. 1. Inhibition of *Staphylococcus aureus* by penicillin (incubation 16 hours at 37°). Galvanometer differences were obtained by subtracting observed readings from that of uninoculated medium control. The penicillin preparation contained 42 Florey units per mg.

Growth in various dilutions of unknown penicillin samples is compared with a standard curve of inhibition run daily side by side with the unknowns. Computation of the potency of the unknown with respect to the standard reference sample of established potency then is an easy matter. Accuracy is that common to microbiological work; namely,  $\pm 10$  to 15 per cent.

Fig. 1 shows the growth response as a function of penicillin concentration. The theoretical basis for the inhibition curve of penicillin and the assay method based on this principle seem established by the logarithmic nature of the inhibition.

Use of a stable reference standard is, of course, imperative. Activities

usually are expressed in terms of Florey units, following the lead of the Oxford group. Penicillin preparations of proved stability should be standardized either directly or indirectly against penicillin preparations of known Florey unitage. Use of a standard eliminates variations due to the culture, media, personal factor, etc. In this work the Oxford strain of *Staphylococcus aureus* has been used.

To tubes containing 5 ml. of various dilutions of penicillin samples are added 5 ml. of sterile, double strength nutrient broth, inoculated just prior to apportionment. For inoculum, 0.4 ml. of a 20 hour nutrient broth culture of the test organism is added to 100 ml. of the double strength broth. Dilutions are made as follows: The original samples of penicillin are diluted with ice-cold sterile 0.02 M phosphate buffer at pH 7.2 so as to contain approximately 0.02 unit per ml. These, together with the standard, are kept in an ice bath until all the samples have been treated similarly. Different amounts, namely 0.5, 1.0, 2.0, 3.0, and 5.0 ml., are then added aseptically to tubes previously sterilized with 4.5, 4.0, 3.0, 2.0, and 0 ml. of buffer, respectively.

Aseptic precautions should be observed throughout. In assaying dry preparations of penicillin, contaminants are not a serious factor owing to the ultimate high dilution and short incubation. With *Penicillium* filtrates or penicillin solutions contaminations may, however, be serious. Such liquids, when possible, should be obtained aseptically and maintained sterile. Otherwise they should be kept cold (0-5°), or saturated with ether or chloroform to minimize contamination. These solvents are without effect on the test organism under these conditions.

After 16 hours (overnight) incubation at 37°, the tubes are shaken, the contents poured into calibrated Evelyn tubes, and the turbidimetric readings obtained. Galvanometer differences (per cent transmissible light) are plotted against penicillin concentration. Three to five levels are run on each unknown, depending on how many can be predicted to fall on the central (three-fourths) region of the curve. A short time (4 hour) assay employing this principle has been developed.

In practice the log curve may be used as reference; only 3 or 4 points are required to define the log curve. Other aspects will be reported later.

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Received for publication, April 6, 1942

## A POSSIBLE VITAMIN D ASSAY TECHNIQUE WITH RADIOACTIVE STRONTIUM\*

Sirs:

It has been suggested<sup>1</sup> that a technique might be developed for the bio-assay of vitamin D by use of radioactive isotopes, activity measurements being made on excreta or biopsy material. Radioactive phosphorus was not used because it exerts an effect similar to that of vitamin D when administered to rats deficient in this vitamin. Furthermore, numerous studies<sup>2</sup> in which radiophosphorus was used on rachitic rats failed to show a practical correlation. Radiocalcium also proved impractical, as pointed out by Pecher,<sup>3</sup> because it is difficult to prepare and to measure. However, Pecher has shown that radiostrontium follows the same course in the body as radiocalcium. We, therefore, fed radioactive strontium and vitamin D to rachitic rats and made activity determinations on the ash of the excreta according to the method of Bale *et al* <sup>4</sup>

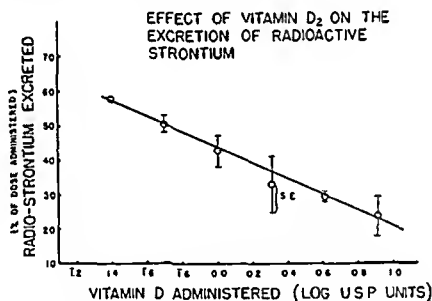


FIG 1 Effect of vitamin D<sub>2</sub> on excretion of radioactive strontium

A  $\text{Sr}^{*}\text{Cl}_2$  solution was administered by stomach tube to standard rachitic rats and to litter mate controls fed an adequate diet since weaning. The animals were kept in metabolism cages following the radiostrontium administration and the feces and urine were collected separately daily. Radioactivity measurements were made on the individual samples of excreta and the results expressed as the per cent of the dose of radio-

\* Communication No 30 from the Laboratories of Distillation Products, Inc

<sup>1</sup> Hickman, D, personal communication

<sup>2</sup> Morgareidge, K, and Manly, M L, *J Nutrition*, 18, 411 (1939) Cohn, W E, and Greenberg, D M, *Proc Am Soc Biol Chem*, *J Biol Chem*, 128, p 461 (1939)

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<sup>3</sup> Pecher, C, *Proc Soc Exp Biol and Med*, 46, 28 (1941)

<sup>4</sup> Bale, W, Haven, F, and LeFevre, M, *Rev Scient Instruments*, 10, 193 (1939)

strontium excreted over a 5 day experimental period. The normal animals were found to excrete 40 to 60 per cent of the dose, while the rachitic negative controls excreted close to 100 per cent. Other groups of rachitic rats received one dose of 1.3 units of vitamin D<sub>2</sub>, given 3 days, 2 days, and 1 day prior to or at the same time as the strontium. These excreted 30, 39, 52, and 56 per cent of the ingested dose respectively. The strontium excreted was roughly proportional to the length of time which elapsed between the vitamin and isotope administration. Vitamin D, therefore, acts rapidly in promoting the retention of strontium (calcium). It may be noted that when sufficient time, *e.g.* 2 or 3 days, is allowed for the vitamin D to become thoroughly assimilated, the radiostrontium excretion of the "healing" animal is below that of the normal. The same amount of vitamin D did not alter the strontium excretion of the normal animal.

In another series, graded doses of vitamin D<sub>2</sub> (0.25 to 8.0 u.s.p. units) were fed to rachitic rats, in groups of two to ten, 48 hours before radiostrontium administration. The animals excreted between 57 and 24 per cent of the dose, as indicated in Fig. 1. This investigation is being continued and extended.

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## ANTISULFONAMIDE ACTION OF ADENINE, 6-AMINOPURINE

Sirs:

The mechanism of the antibacterial action of the sulfonamides remains unexplained. One approach to the solution of this problem is through the study of compounds exerting an inhibitory effect on sulfonamide action. Various agents have been described as having such activity: peptones, purulent exudates,<sup>1</sup> *p*-aminobenzoic acid,<sup>2</sup> methionine,<sup>3</sup> cozymase,<sup>4</sup> and urethane.<sup>5</sup> Both cozymase and urethane have been questioned as antisulfonamide<sup>6,7</sup> compounds. The concept of specific competition between sulfonamide drugs and essential metabolites was proposed by Fildes.<sup>8</sup> The possibility that nucleic acid of yeast might contain an inhibitor of sulfonamide action against *Staphylococcus aureus* was suggested by Strauss *et al.*<sup>6</sup>

Adenine sulfate, EK No. 1645, was found to be non-toxic at levels of 1.0 gm. per kilo in mice. The toxic level of sulfadiazine and sulfathiazole was not altered by the concomitant oral administration of adenine sulfate. The chemotherapeutic efficacy of sulfanilamide (2 mg. per gm.) and sulfadiazine, sulfapyridine, and sulfathiazole (all at 4 mg. per gm.) was nullified by adenine sulfate (0.8 mg. per gm.). All tests were carried out *in vivo*. Mice (700) were injected with 0.1 cc. of a 24 hour broth culture of *Streptococcus hemolyticus*. With no sulfonamide, 100 per cent deaths occurred in 24 hours. With sulfonamides at the indicated levels, the percentage of deaths ranged from 0 to 30. With sulfonamides at the indicated levels but with added adenine sulfate, death rates ranged from 90 to 100 per cent. *p*-Aminobenzoic acid (0.8 mg. per gm.) exerted less antisulfonamide action than did adenine sulfate at the same level when both were tested against sulfadiazine or sulfathiazole. Guanine (2-amino-6-oxypurine) and uracil (2,6-dioxypyrimidine) exerted no antisulfonamide action at levels as high as 2.0 mg. per gm. against the sulfonamides at the above dosages.

Adenine was established as an essential nutrient substance for hemolytic streptococci by Pappenheimer and Hottle,<sup>9</sup> and its function in combination

<sup>1</sup> MacLeod, C. M., *J. Exp. Med.*, **72**, 217 (1940).

<sup>2</sup> Woods, D. D., *Brit. J. Exp. Path.*, **21**, 74 (1940).

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with ribosephosphoric acid ester as part of the coenzymes, codehydrogenase, and cophosphorylase is well known. These facts suggest as an explanation of the action of sulfonamides against streptococci their interferences with the normal utilization of adenine, an essential growth factor functioning in vital enzyme systems.

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# THE INFLUENCE OF FASTING, EPINEPHRINE, AND INSULIN ON THE DISTRIBUTION OF ACID-SOLUBLE PHOSPHORUS IN THE LIVER OF RATS\*

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The normal distribution of phosphorus compounds in the liver and changes of these compounds in different conditions have been little studied. Cori and Goltz (1) described an increase of the inorganic P and easily hydrolyzable organic P fractions in the livers of mice consequent to the administration of insulin. In animals subjected to various experimental procedures, Flock and her coworkers (2) found no change in the distribution of phosphorus in the livers except a general decrease in all fractions in the fatty livers obtained after the administration of a high fat diet.

The studies here reported are part of a series dealing with the distribution and metabolism of phosphorus compounds in various tissues.

## EXPERIMENTAL

Rats from our stock colony weighing  $222 \pm 45$  gm. were used. The animals designated "fed" had access to food up to the time of the administration of insulin or epinephrine, while the fasted animals were deprived of food for 16 to 18 hours prior to the beginning of the experiments. Insulin was administered subcutaneously in doses of 2 or 5 units per animal.<sup>1</sup> Epinephrine was injected subcutaneously, in doses of 0.2 mg. per kilo of body weight.

1 hour after the subcutaneous injection of either hormone the animals were killed under deep amytal anesthesia by decapitation and blood was collected for analyses. The liver was removed quickly and dropped into a dry ice-ether mixture. After the tissue was thoroughly frozen, it was powdered in a chilled steel mortar. Approximately 2 gm. portions of powdered liver were added to 20 cc. of ice-cold 5 per cent trichloroacetic acid in tared centrifuge tubes and the tubes were then reweighed. The

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<sup>1</sup> Since no difference was found between findings among rats given 2 units and 5 units, the data were not separated according to dosage.

tubes were shaken vigorously for 10 minutes, centrifuged, the supernatant fluid removed, and the residue reextracted with 5 cc. of 5 per cent trichloroacetic acid. The wash fluid was added to the first extract and the combined fluids were filtered to remove remaining particles.

The method of Fiske and Subbarow (3) was used for the determination of the inorganic P, the total acid-soluble P, and the easily hydrolyzable P (that liberated by 8 minutes heating with 1 N sulfuric acid in a boiling water bath). The organic phosphorus not liberated by 8 minutes acid hydrolysis was designated "rest" phosphorus. Liver glycogen was determined in portions of the frozen liver by the method of Good, Kramer, and Somogyi (4). Blood sugar was determined by the micromethod of Somogyi (5).

Statistical analysis of the data was made by Mrs. E. W. Brown, in accordance with the methods of Fisher (6).

TABLE I  
*Influence of Fasting, Insulin, and Epinephrine on Liver Glycogen  
and Blood Sugar of Rats*

		No. of rats	Liver glycogen; mean and S.E.*	Blood sugar; mean and S.E.*
			per cent	mg. per 100 gm.
Fed	Control	10	$3.67 \pm 0.44$	$116 \pm 4.6$
	Insulin	14	$2.56 \pm 0.18$	$49 \pm 3.1$
Fasted	Control	9	$0.11 \pm 0.04$	$84 \pm 3.8$
	Epinephrine	11	$0.31 \pm 0.05$	$181 \pm 6.4$
	Insulin	10	$0.01^\dagger$	$24 \pm 1.1$

\* Standard error of the mean.

† No statistical description of this value was attempted, since all concentrations were at the lowest limit of measurement by the method used.

### Results

Data summarized in Table I indicate that insulin produced a decreased blood sugar and liver glycogen in both fed and fasted animals. Epinephrine produced an increased blood sugar and liver glycogen in the fasted animals. Those changes were found to be statistically significant.

The data summarized in Table II indicate that insulin produced a marked and consistent increase in the total acid-soluble P concentration in the livers of both fed and fasted rats. This increase appeared to be due for the most part to a rise in the organic rather than inorganic fractions; both the easily hydrolyzable and the rest fractions in the livers of both groups increased considerably, while the inorganic P increased significantly only in the fasted animals. Following the injection of epinephrine there was little if any change perceptible: there appeared to be a slight shift (approaching

statistical significance) of inorganic P into the rest P fraction, while the total P and easily hydrolyzable P remained unchanged. In the livers of rats deprived of food 16 to 18 hours the inorganic P appeared to be increased

TABLE II

*Influence of Fasting, Insulin, and Epinephrine on Distribution of Acid-Soluble Phosphorus in Livers of Rats*

		No. of rats	Total acid soluble P, mean and s.e.*	Inorganic P, mean and s.e.*	Easily hydrolyzable P, mean and s.e.*	"Rest" P, mean and s.e.*
			mg per 100 gm	mg per 100 gm	mg per 100 gm	mg per 100 gm
Fed	Control	10	100.5 ± 1.7	17.5 ± 0.7	18.3 ± 0.4	64.7 ± 1.5
	Insulin	14	116.8 ± 1.7	18.2 ± 0.7	22.2 ± 0.7	76.3 ± 1.8
Fasted	Control	9	97.0 ± 2.0	21.9 ± 0.4	16.2 ± 0.4	59.1 ± 1.2
	Epinephrine	11	98.4 ± 1.4	20.2 ± 0.6	16.1 ± 0.7	62.1 ± 0.7
	Insulin	10	112.2 ± 1.6	24.2 ± 0.6	19.4 ± 0.7	68.6 ± 1.2

\* Standard error of the mean

TABLE III

*Significance of Differences between Mean Concentrations of Acid-Soluble Phosphorus in Livers of Rats*

		Total acid soluble P		Inorganic P		Easily hydrolyzable P		"Rest" P	
		Difference	p*	Difference	p*	Difference	p*	Difference	p*
		mg per 100 gm		mg per 100 gm		mg per 100 gm		mg per 100 gm	
Fed	Insulin vs control	+16.3	0.001	+0.8	0.05-0.4	+3.9	0.001	+11.6	0.001
Fasted	" "	+15.2	0.001	+2.3	0.01-0.001	+3.2	0.001	+9.6	0.001
	Epinephrine vs control	+1.4	0.06-0.05	-1.7	0.05-0.02	-0.1	0.90	+3.0	0.05-0.02
No treatment	Fasted vs fed	-3.5	0.30-0.20	+4.4	0.001	-2.1	0.001	-5.6	0.02-0.01
Insulin	" "	-4.6	0.10-0.05	+6.0	0.001	-2.8	0.01	-7.7	0.001

\* p = percentage chance that a deviation as great or greater than that observed would arise by chance alone, any value of p 0.05 or less is usually accepted as indicating a significant difference (Fisher (7))

at the expense of the organic P without change in the concentration of the total acid-soluble P.

The differences and estimates of significance of the differences between values for the various phosphorus fractions in the livers of the several groups are summarized in Table III.

## DISCUSSION

*Effect of Fasting*—In the livers of fasted rats the concentration of inorganic P increased at the expense of both the easily hydrolyzable and the rest phosphorus fractions. No reasonable explanation for this shift is apparent. Such a change might be thought to be related to glycogenolysis, except that after an 18 hour fast the glycogen has been reduced to a low concentration in the liver for several hours and it is probable that we are dealing here with a steady state rather than with changes associated with a progressive diminution in the concentration of glycogen.

*Effects of Insulin and Epinephrine*—Suggestions which have been made to explain the decreases of inorganic P in the plasma which follow the administration of insulin and epinephrine include claims that the phosphorus leaving the plasma enters the erythrocytes (8, 9), muscle (10), or liver.

When careful attention is paid to changes in the volume of cells in the blood, there appears to be little or no evidence that the inorganic P leaving the plasma is to be accounted for by an increase of organic acid-soluble P in the erythrocytes (Kerr (11); also Guest, unpublished studies).

The claim that the decrease in inorganic P in the plasma following the injection of insulin could be attributed to an increase of the total acid-soluble P in the muscles (10) was not supported by the results of later investigations (12, 13). More definite conclusions with reference to the uptake of phosphorus by the muscles can scarcely be reached now, because the muscles contain in the acid-soluble form approximately 100 times the amount of inorganic P that is in the blood. Even if all the inorganic P of the plasma were transferred to the muscles, the resultant change in concentration of acid-soluble P in the muscles would be below the limits of measurement by analytical methods available today. Changes found in one of the fractions of the acid-soluble phosphorus in the muscles would not be evidence of such a process in the face of constant values for the total acid-soluble P.

Our data indicate that increases of acid-soluble P found in the livers of rats following the injection of insulin were approximately equal to the decrease of inorganic P in the extracellular fluid.<sup>2</sup> Such findings suggest

<sup>2</sup> The average concentration of inorganic P in the plasma of the control rats was 5.93 mg. per 100 cc., and 4.27 mg. per 100 cc. in the plasma of the rats treated with insulin, a fall of 1.66 mg. per 100 cc. Assuming that the extracellular fluid amounts to 30 cc. per 100 gm. of rat, the amount of phosphorus that disappeared from the extracellular fluid was calculated to be  $1.66 \times 0.30 = 0.50$  mg. of P per 100 gm. of rat. In our fasted animals the liver weighed 2.8 gm. per 100 gm. of rat and the acid-soluble phosphorus increased by 15.2 mg. per 100 gm. of liver. From this the amount of phosphorus taken up by the liver was calculated to be 0.43 mg. per 100 gm. of rat,

that insulin influences the level of inorganic P in the blood plasma at least in part through its effect on the liver.

To what extent the effects of insulin, glucose, and epinephrine are interdependent remains a subject for further speculation. It has been suggested (12) that the effect of insulin on the plasma P was mediated by epinephrine, since increases in hexose monophosphate in muscle appeared to be due exclusively to epinephrine (14, 15). This suggestion seems to be untenable, however, because epinephrine fails to produce a drop of plasma P in depancreatized animals (8, 16) and because epinephrine does not produce any significant increase of liver phosphate in the normal animal. The fact that the plasma P does not decrease when epinephrine is given to depancreatized animals constitutes indirect evidence that insulin plays a rôle in the effect of epinephrine on plasma P in normal animals. The decrease of plasma P that follows the injection of epinephrine in normal animals might be thought to be produced by a reflex insulin secretion, but our data on the effect of epinephrine do not show the increase of total acid-soluble P in the liver that would be expected if this were true. It is of course possible that plasma phosphorus enters muscle following the injection of epinephrine. It has been suggested (17) that one of the effects of epinephrine is to cause a shift of plasma potassium into the muscle; if so, there might be a concomitant shift of phosphorus. However, it is not clear how the absence of insulin (*i.e.*, pancreatectomy or diabetes) might modify such a mechanism.

When insulin is administered with glucose to normal animals, a greater decrease of plasma inorganic P is observed than with either alone (18). The findings of Fenn (19) suggest the possibility that rapid glycogen deposition brought about by the administration of glucose entails a withdrawal of inorganic P from the blood plasma. Such a mechanism may supplement the insulin effect.

#### SUMMARY

Insulin appears to cause a decrease of inorganic phosphorus in the blood plasma in part through its action on the liver.

Following the injection of insulin in fed and fasted rats the concentrations of inorganic and organic acid-soluble phosphorus in their livers increased.

Injections of epinephrine in fasted rats were followed by little if any

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representing 86 per cent of that which disappeared from the extracellular fluid. It is of course possible that changes in the balance between the liberation and excretion of waste inorganic phosphate and movements of phosphate between various organs may occur under the influence of insulin; such changes are difficult to evaluate.

significant change in the distribution of acid-soluble phosphorus in the livers.

In the livers of rats fasted 16 to 18 hours the inorganic phosphorus increased without significant change of the total acid-soluble phosphorus.

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# THE BEHAVIOR OF LIPIDS DURING AUTOLYSIS OF LIVER AND BRAIN\*

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The apparent similarity between the process of fatty infiltration or degeneration *in vivo* and the appearance of fat droplets in tissue cells during sterile incubation *in vitro*, first noted by Hauser (1) in 1886, stimulated numerous biochemical and histological studies of the behavior of lipids during autolysis. About the same time Kraus (2) found that the quantity of ether-extractable material in liver did not change during 2 to 4 weeks incubation, but in subsequent investigations (3-18) of total lipids or fatty acids highly conflicting results were obtained. The fate of cholesterol during autolysis of tissues is equally uncertain. Different investigators (4, 15, 19-27) found increases, interpreted to be the result of synthesis, decreases, considered to represent degradation, or no change in the quantity of cholesterol or of the unsaponifiable fraction. Artom (24) observed large increases or decreases which he related to the state of nutrition of the animal from which the tissue was taken for autolysis. In most of these investigations sterile, *minced* tissue, usually liver, was incubated at body temperature, with analysis of aliquots for total lipids or cholesterol at the start of, and at varying intervals during the autolysis.

In the present study it was shown that no synthesis or destruction of cholesterol occurs during autolysis of *intact* slices or pieces of liver. However, there is a large increase in the percentage of cholesterol in dry tissue. This result, which was observed in the course of a study of cholesterol esterase (28), at first appeared to support the contention of several workers (4, 21-24) that autolyzing liver has the ability to synthesize cholesterol; but this is not the correct explanation. There is no increase in the total *quantity* of cholesterol; the *concentration* increases because a large decrease in the dry weight of the tissue is not accompanied by an equivalent decrease in the amount of cholesterol. On the contrary most or all of the cholesterol originally present is retained in the pieces of liver which survive incubation. The same process was probably responsible for the finding by

\* A preliminary report of this investigation was presented before the American Chemical Society at St. Louis, April, 1941.

Waldvogel (4) and by Garnier and Sabaréanu (5) of an increase in percentage of lipids during incubation of pieces of liver.

In contrast with the behavior of cholesterol there is a definite decrease in the total amount of phospholipids in pieces of liver during autolysis, probably as a result of the decomposition which previous investigators, with one exception (29), found (30-32) to accompany autolysis of minced tissues. However, this decrease in *quantity* is proportionally less than the decrease in dry weight of tissue, with the result that the *concentration* increases; phospholipids, like cholesterol, are retained and concentrated in the intact pieces of liver which survive autolysis.

A similar process could not be demonstrated in brain during incubation for 1 day. It is sometimes stated that nervous tissue does not autolyze (33), but there is considerable evidence (34-38) to show that it does so at an appreciable rate. It is possible that the period of incubation employed in the present investigation was too short, but Trzebinski (36) reported histological changes in spinal cord after autolysis for only 4 to 6 hours and Soula (37) found up to 20 per cent autolysis of brain, as measured by proteolysis, in 1 day.

#### EXPERIMENTAL

*Procedure with Liver Slices*—Normal, adult rats were killed by decapitation and the blood was drained as completely as possible. The liver was removed immediately and sliced with a razor blade; the slices were washed with four portions of Tyrode's solution. Control slices were blotted on hard filter paper, weighed into dry 5 cc. volumetric flasks, boiled with alcohol-acetone (1:1), and analyzed for cholesterol by the method of Schoenheimer and Sperry (39) as modified by Sperry (40). Experimental slices were incubated in test-tubes for 1 or 2 days in an excess of Tyrode's solution containing 0.0001 per cent sodium ethyl mercurithiosalicylate as a preservative. They were then rinsed to remove the adherent debris, which formed in copious amounts, blotted, weighed, and analyzed in the same way. Dry weight was calculated from determinations of water content in replicate control and experimental samples which were dried to constant weight at 110-115°.

In seven series of from four to nine replicate determinations of cholesterol (thirty-seven in all) by this procedure the standard deviation of the percentage deviation from the mean was 4.12 per cent. That incompleteness of extraction was not responsible for this relatively high error was shown in two ways. (a) The residues from nine such extractions of fresh liver slices were dissolved in alcoholic potassium hydroxide and analyzed after neutralization with alcoholic hydrochloric acid. A negligible quantity of cholesterol was found. (b) Replicate samples were analyzed by the extraction

method and by the procedure described under (a). In thirteen such comparisons the average cholesterol concentration found by extraction was 0.199 per cent and by the procedure involving solution in alkali, 0.194 per cent. At the time most of the experiments with liver slices were carried out it was not realized that they take up considerable quantities of water from "physiological" saline solutions (41). Since replicate samples remained in Tyrode's solution for variable periods before incubation or analysis, this factor probably accounts in large part for the relatively high variability.

*Experiments with Liver Slices*<sup>1</sup>—In four experiments carried out in quadruplicate or quintuplicate the concentration of cholesterol in liver slices increased from 0.84, 0.93, 0.96, and 0.78 to 1.20, 1.59, 1.90, and 1.69 per cent dry weight respectively during incubation for 1 day. That this result, which was obtained consistently on incubation of slices or pieces of liver (Table I), did not represent cholesterol synthesis was shown by incubating weighed slices in 5 cc. volumetric flasks and analyzing the entire contents. The average percentage of cholesterol in five control samples was 0.188 and in five incubated samples, 0.194 (on the basis of the fresh weight). In this experiment it was possible to add enough Tyrode's solution barely to cover the slices, as more would have interfered with the analysis. Hence weighed slices were incubated in centrifuge tubes (high speed, International Equipment Company) under an excess of solution. The slices and debris were isolated by centrifuging, dissolved in alcoholic alkali, transferred to volumetric flasks, neutralized, and analyzed. The average percentage of cholesterol in four control samples was 0.212 and in four incubated samples, 0.210. This experiment was repeated with liver sliced in a moist box and weighed for analysis or incubation without contact with a solution (41). The recovery of cholesterol in the centrifuged slices and debris after 2 days incubation averaged 97 per cent in six samples from two livers.

To obtain a more complete picture of the process by which the concentration of cholesterol increases during autolysis of liver slices, balance experiments (Experiments 1 and 2, Table I) were carried out in quadruplicate and triplicate respectively. In Experiment 1 the slices were washed as described above, blotted, and weighed into centrifuge tubes in which they were incubated in an excess of Tyrode's solution; in Experiment 2 the liver was sliced and the control and experimental slices were weighed without contact with a solution (41). After incubation the intact slices were rinsed and analyzed as described. The debris was isolated from the supernatant solution and rinsings by centrifuging, and analyzed separately. The cholesterol represented by the decreases of 6 and 12 per cent respectively in the

<sup>1</sup> Most of the experiments with tissue slices were carried out in the Chemical Laboratory of the Babies Hospital with the aid of a grant from the Carnegie Corporation of New York.

content of the slices (Table I) was recovered quantitatively in the débris. In Experiment 2 the total recovery in slices and débris was 99.2, 102.2, and 98.6 per cent in the three replicate samples respectively.

*Procedure with Pieces of Liver*—In certain of our experiments (Nos. 3 to 8, Table I) the liver was cut into pieces weighing from 0.5 to 1.0 gm. in order to reduce the amount of surface and to prevent to some extent the

TABLE I  
*Behavior of Cholesterol during Autolysis of Liver*

Experiment No.	Procedure*	Time	Cholesterol in dry liver		Dry weight of sample			Total cholesterol in sample		
			Start	End	Start	End	Decrease	Start	End	Decrease†
		days	per cent	per cent	mg.	mg.	per cent	mg.	mg.	per cent
1	A	1	0.78	1.61	83	38	54	0.65	0.61	6
2	"	2	0.87	1.42	58	31	47	0.50	0.44	12
3	B	4	0.76	1.83	486	189	61	3.69	3.46	6
4	"	1	0.72	1.31	1227	674	45	8.83	8.83	0
5	"	1	0.75	1.18	1259	814	35	9.44	9.61	-2
6	"	1	0.74	1.31	1076	602	44	7.96	7.89	1
7	"	1	0.75	1.15	1026	653	36	7.70	7.51	2
8	"	1	0.76	1.26	1015	625	38	7.71	7.88	-2
9†	C	4	0.90	1.37	249	159	36	2.24	2.18	3
10†	"	4	0.90	1.39	177	113	36	1.59	1.57	1
11	"	2	0.81	1.07	297	209	30	2.41	2.24	7
12	D	4	0.77	1.14	302	204	32	2.33	2.33	0
13	"	2	0.74	1.06	438	282	36	3.24	2.98	8
14	"	5	0.77	1.18	310	203	35	2.39	2.40	0
15	"	4	0.74	1.16	521	313	40	3.86	3.63	6
16	"	3	0.74	1.00	362	258	29	2.68	2.58	4
17	E	2	1.02	1.43	296	192	35	3.02	2.75	9
18	"	2	0.73	1.38	342	198	42	2.50	2.73	-9
19	"	1	0.65	1.15	332	185	44	2.16	2.13	1
20	"	3	0.69	1.39	222	142	36	1.53	1.97	-29

\* Procedure A, slices in Tyrode's solution; Procedure B, pieces in 0.9 per cent sodium chloride solution; Procedure C, pieces with nothing added; Procedure D, pieces in rubber bags in abdominal cavity; Procedure E, pieces in abdominal cavity.

† A minus sign designates an increase.

‡ These two experiments were carried out on different samples from the same rat.

formation of débris, which was extensive with the liver slices. The liver pieces were incubated in 0.9 per cent sodium chloride solution containing sodium ethyl mercurithiosalicylate, rinsed, blotted, and weighed in the same manner as the liver slices.

For comparison with this technique in another series of experiments (Nos. 9 to 16, Table I) pieces of liver were removed under sterile conditions and

incubated at 38° in dry sterile tubes or placed in sterile rubber finger cots and returned to the abdominal cavity of the rat, which served as an incubator (see below). In no case was there any evidence of bacterial growth. After the pieces had been incubated for the desired length of time they were removed from the fluid and débris which formed, and treated as mentioned above.

For determination of cholesterol, control and incubated pieces of liver were minced and analyzed in duplicate with the aid of a small sampling tube with a plunger as described in a former paper (40). In 78 such determinations the standard deviation of the percentage deviation from the mean was 1.73 per cent. From the weights of the samples before and after incubation and the percentages of water, determined by drying aliquots of the minced tissue in an oven, the decrease in dry weight (Table I) was calculated.

*Experiments with Pieces of Liver*—In all of the experiments (Nos. 3 to 16, Table I) carried out with the techniques described in the preceding section most or all of the cholesterol was retained in the pieces of liver despite a large decrease in the dry weight of the liver. Although the formation of débris appeared to be proportionally less than in the experiments with slices, it was still large. Microscopic examination of incubated tissue showed large numbers of intact or partially disintegrated cells which were either free or loosely attached at the periphery; the débris was presumably composed of such disintegrated tissue. Since most of the cholesterol originally present was found in the intact pieces of liver, the cholesterol of the disintegrated cells apparently had been adsorbed in some way by the remaining tissue.

*Histological Studies*—In an attempt to determine whether cholesterol is thus adsorbed one of us (W. M. C.) carried out histological studies of slices and pieces of liver which had been incubated in saline solution side by side with the experimental samples. The findings having a bearing on the interpretation of the biochemical results were (a) considerable disintegration along the periphery, (b) considerable shrinkage in size of the parenchyma cells with an associated enlargement of sinusoidal spaces, (c) little evidence of destruction of cells except at the periphery, (d) a marked increase in intra- and intercellular globules staining with osmic acid, scarlet red, and other fat stains, particularly in the sinusoidal blood spaces, and (e) the presence of many small, needle-shaped, anisotropic crystals, some of which took fat stains, throughout the tissue, both within and outside the cells. Most of these changes have been reported by one or more of the investigators (1, 4, 21, 42-46) who have studied autolyzed tissue under the microscope. Attempts to visualize cholesterol by Schultz's method (sulfuric and acetic acids on frozen sections) or with digitonin met with

failure. Crystals which were probably cholesterol digitonide were seen, but it was impossible to differentiate them with certainty from the crystals mentioned above. The presence of needle-shaped crystals in autolyzed liver was noted by Hauser (1), who called them "fat" crystals without explanation, and by Aschoff (quoted by Waldvogel (4)). They do not seem to have been studied further and their nature is not known.

*Experiments in Vivo*—Borger *et al.* (47) showed that the process occurring in artificial tissue infarcts, such as the ligated kidney, is quite different from that of autolysis. After a few hours the infarcted tissue becomes alkaline instead of acid, and the lactic acid and ammonia concentrations are not elevated as in autolyzing tissue. Severed kidneys placed in the abdominal cavity are subject to the same changes as ligated kidneys. However, if a non-permeable rubber membrane is tied around the kidney, it autolyzes as in the incubator *in vitro*.

It was of interest to study the behavior of lipids under the foregoing conditions. With sterile technique laparotomy was performed in rats under ether anesthesia and the pedicle of one of the large lobes of the liver was ligated with a thick soft cord. The entire lobe was severed and divided along the anterior-posterior axis into two approximately equal portions, of which one was analyzed as a control and the other weighed on a sterile watch-glass and returned to the lower part of the abdominal cavity. The incision was closed. After 1 to 4 days the rat was killed and the piece of liver was removed, blotted on filter paper, weighed, and analyzed as before. The results (Experiments 17 to 20, Table I) show the same retention of cholesterol found in autolyzed liver. The increase in cholesterol content in Experiment 20 may have been the result of absorption of cholesterol from abdominal fluids.

A portion of the minced tissue was suspended in a little water and the pH was determined with the glass electrode. The values ranged from 7.8 to 8.3 in confirmation of Borger's (47) finding. With the same technique the pH of the control liver samples ranged from 6.6 to 6.8.

In another series of experiments (Nos. 12 to 16, Table I) the procedure was exactly the same except that the piece of liver was tied firmly in a sterile rubber finger cot before it was returned to the abdominal cavity (see above). The pH ranged from 5.0 to 6.1 in these experiments.

*Behavior of Phospholipids in Autolyzing Liver*—In some of the foregoing experiments, in which pieces of liver autolyzed in 0.9 per cent sodium chloride solution, phospholipids were also determined (Table II). Duplicate or triplicate samples of minced liver (control and autolyzed) were boiled with alcohol-ether (3:1) in volumetric flasks and phosphorus was determined in aliquots of the filtrates by a micro modification (48) of the method of Fiske and Subbarow (49). Phospholipid was assumed to be 25 times the phos-

TABLE II

*Behavior of Phospholipids during Autolysis of Liver*

Liver was incubated 1 day in 0.9 per cent sodium chloride.

Experiment No.	Phospholipid in dry liver		Dry weight of sample			Total phospholipid in sample		
	Start	End	Start	End	Decrease	Start	End	Decrease
	per cent	per cent	mg.	mg.	per cent	mg.	mg.	per cent
5	11.2	16.3	1227	674	45	137	110	20
6	11.4	14.0	1259	814	35	144	114	21
7	12.7	15.5	1076	602	44	137	93	32
8	11.6	13.3	1026	653	36	119	87	27
9	12.4	15.2	1015	625	38	126	95	25
32	12.5	15.4	1149	657	43	144	101	30

TABLE III

*Behavior of Cholesterol during Autolysis of Brain*

Brain was incubated 1 day in 0.9 per cent sodium chloride.

Experiment No.	Cholesterol in dry brain		Dry weight of sample			Total cholesterol in sample		
	Start	End	Start	End	Decrease	Start	End	Decrease
	per cent	per cent	mg.	mg.	per cent	mg.	mg.	per cent
6*	10.2	10.2	247	228	8	25.2	23.3	8
7	8.1	7.8	225	204	9	18.2	15.9	13
8	8.1	7.8	234	228	3	19.0	17.8	6
31†	9.4	9.9	522	481	8	49.1	47.6	3

\* The percentage of water determined in the control sample (63.4) was obviously in error. The average value obtained in the other experiments (75.1) was used in calculating the data.

† Two brains were pooled in this experiment.

TABLE IV

*Behavior of Phospholipids during Autolysis of Brain*

Brain was incubated 1 day in 0.9 per cent sodium chloride.

Experiment No.	Phospholipid in dry brain		Dry weight of sample			Total phospholipid in sample		
	Start	End	Start	End	Decrease	Start	End	Decrease
	per cent	per cent	mg.	mg.	per cent	mg.	mg.	per cent
6*	23.4	22.6	247	228	8	58	52	10
7	22.5	22.7	225	204	9	51	46	10
8	23.6	21.7	234	228	3	55	49	11
31†	24.2	23.1	522	481	8	126	111	12
32†	24.2	24.1	407	393	3	98	95	3

\* Cf. foot-note to Experiment 6, Table III.

† Two brains were pooled in these experiments.

phorus. The standard deviation of the percentage deviation from the mean was 2.37 per cent in thirty-six such determinations.

*Behavior of Cholesterol and Phospholipids during Autolysis of Brain*—Rat brains were divided along the mid-line into two parts as nearly equal as possible. One was weighed, incubated in 0.9 per cent sodium chloride for 1 day, turned over on filter paper to remove excess fluid, reweighed, ground up to a paste in a mortar, and analyzed for cholesterol, phospholipids, and water as described for liver. The other part was analyzed in the same way as a control immediately after removal from the animal. It was stated (40) that the procedure proposed for cholesterol determination in the liver is not applicable to brain, but it was found later that fairly good results can be obtained if the brain is thoroughly ground to a paste. The standard deviation of the percentage deviation from the mean in eighteen duplicate determinations was 2.90 per cent for cholesterol and 2.02 per cent for phospholipid.

The results (Tables III and IV) give no indication of a process in brain like that found in liver. The small decreases in dry weight and in the lipids were of the same order of magnitude in most experiments and were probably for the most part mechanical, as some tissue adhered to the filter paper in removing the excess fluid. Another source of error was the impossibility of dividing the various structures of the brain, which differ considerably among themselves in lipid and water concentration, into exactly equal parts.

#### DISCUSSION

In this investigation we have demonstrated conclusively that cholesterol is neither synthesized nor destroyed during autolysis of slices or pieces of liver. This result is in apparent disagreement with the findings of previous investigators (21-27) who reported large increases or decreases in the cholesterol content of minced tissues during incubation. It is unlikely, though possible, that preparations in which the cells are largely destroyed can carry out reactions which the intact liver is unable to accomplish.

The finding that cholesterol is retained in slices or pieces of liver, even though a large part of the tissue solids is lost in the process of autolysis, explained most simply by the assumption that as the protein of the liver is broken down and the soluble end-products diffuse out, the insoluble cholesterol remains behind. The shrinkage and the lack of destruction of liver cells, except at the periphery, are in accord with this hypothesis. The possibility remains that cholesterol set free during the disintegration of tissue at the periphery, a process which appears microscopically to be of considerable magnitude, is adsorbed by the intact tissue.

Artom (30) found an average decrease in phospholipids of 33 per cent



during incubation of minced liver for 1 day. Since the decrease was no larger in the present study (average 26 per cent, Table II) it may be deduced that no undissociated phospholipid diffused from the pieces of liver. Recently Chaikoff *et al.* (50) reported a decomposition of about 30 per cent of the phospholipids of liver slices in 6 hours. The close agreement of this result with those of Artom and ourselves after incubation for 1 day suggests that autolytic breakdown of phospholipid in liver goes to completion within 6 hours. The fat-staining globules, which appeared, particularly in the sinusoidal blood spaces, may have represented the end-products of phospholipid decomposition.

#### SUMMARY

Cholesterol is neither synthesized nor destroyed during sterile incubation of slices or pieces of rat liver for 1 to 4 days, either in saline solution or with nothing added.

Although a large loss of tissue solids occurs during autolysis of liver, most or all of the cholesterol originally present is retained in the intact tissue, with a resulting marked increase in concentration. The mechanism of this process is discussed.

Cholesterol is concentrated in the same way in severed pieces of liver left free in the abdominal cavity.

There is a considerable decomposition of phospholipids in autolyzing liver, but this is proportionally less than the decrease in dry weight of tissue, with the result that phospholipids, like cholesterol, are concentrated in the intact pieces of liver which survive autolysis.

No significant changes in cholesterol or phospholipids occurred in brain during incubation for 1 day.

Histological changes in autolyzed liver are described briefly.

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# STUDIES ON PLASMA PHOSPHATASE ACTIVITY AND ON BLOOD PHOSPHOLIPIDS IN RATS WITH OBSTRUCTIVE JAUNDICE

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Previous work has indicated the close relationship between the plasma phosphatase activity and the fat metabolism of normal albino rats (1). The change in plasma phosphatase activity of rats was found to be specifically connected with the ingestion of certain unsaturated fatty acids (1). The extension of our experiments to rats in which fat resorption was inhibited by the obstruction of the bile duct should be, therefore, of value to the better understanding of this complex mechanism.

## EXPERIMENTAL

The plasma phosphatase determinations were carried out as described in our previous papers (1, 2). Only the full activities (with Mg) are given, since the initial activities (without Mg) run parallel to these.

*Ultramicromethod for Determination of Blood and Plasma Phospholipids (Alcohol-Ether-Soluble)*—For the determination of blood and plasma phospholipids the method of Boyd (3) was modified to permit estimations in 4 c.mm. of blood or 8 c.mm. of plasma. The few drops of blood necessary for the determination were taken from the tail of the rat and handled as previously described (1). Micro test-tubes (25 mm. long and 4 mm. inside diameter) containing 200 c.mm. of alcohol-ether mixture (3 parts of 95 per cent alcohol to 1 part of ether) were immersed in ice, then 8 c.mm. of plasma or 4 c.mm. of whole blood were added with a capillary pipette, and the tubes were closed with rubber caps. The mixing of the blood samples in the solvent was carried out by means of small glass beads, filled with iron powder, magnetically agitated (4). To complete the extraction of the phospholipids a shaking device was constructed.

The micro test-tubes containing the blood samples and solvent were uncapped and placed in the holes of a brass disk 7 cm. in diameter, the tubes resting on a rubber cushion. The upper disk, covered on the under side with a sheet of synthetic rubber  $\frac{1}{8}$  inch thick (purchased from the Anchor Packing Company, Philadelphia, No. 6900), was tightly screwed over the openings of the micro test-tubes. Synthetic rubber had the advantage of being insoluble in the solvent used for extraction. The

shaking device was mounted at an angle of  $45^\circ$  and attached to a phonograph motor turned to a horizontal position (50 R.P.M.) During rotation, the alternating movements of the air bubble above the solvent and the stirring bead effected a thorough mixing. Half an hour was found sufficient for complete extraction. A longer extraction period did not increase the phospholipid content of the solvent but for the sake of safety a 1 hour extraction period was used in all experiments.

After the extraction period the micro test-tubes were removed, closed with rubber caps, and centrifuged. 50 to 150 c.mm. of the supernatant solvent, depending on the phospholipid content of the extract, were transferred to a clean test-tube (35 mm. long and 3.5 mm. inside diameter). In evaporating such a small amount of solvent, care must be taken that no loss occurs owing to vigorous boiling and that the residue remains collected at the bottom of the test-tube. For this purpose the micro test-tubes containing the alcohol-ether extract were placed in holes bored in a copper block (5 mm. deep), and the latter was placed on an electric hot-plate, the temperature of which could be regulated and read by means of a thermometer inserted in a horizontal bore of the block. The whole arrangement was covered with a glass funnel to prevent contamination by dust.

The temperature first was raised to  $60^\circ$ , which permitted a relatively rapid evaporation without actual boiling. The vaporized solvent condensed on the walls of the micro test-tubes and redissolved any lipids which coated the tubes during evaporation. With this procedure it was possible to collect the lipids at the bottom of the test-tubes. After about 1 hour the temperature was raised to  $70^\circ$ , in another hour to  $80^\circ$ , and as soon as the tubes were dry to  $140^\circ$  to drive out all traces of solvent, the time required for the whole procedure being about 5 to 6 hours. After cooling, 5 c.mm. of 70 per cent perchloric acid were pipetted into the tubes containing the dry lipid residue and heated to  $200^\circ$  on the copper block until the reaction mixture was clear, indicating that oxidation was complete. The time required for the oxidation was about 30 minutes. The volume of perchloric acid lost during the heating period was negligible. The reaction mixture after cooling was now ready for the colorimetric determination of phosphorus.

After the addition of 15 c.mm. of distilled water, the color was developed by adding 7 c.mm. of 5 per cent ammonium molybdate (made up without sulfuric acid because of the perchloric acid already present in the reaction mixture) and 5 c.mm. of aminonaphtholsulfonic acid solution, as described in our previous paper, and the comparison of colors was carried out as before (1). The standard phosphate solutions contained the same amount of perchloric acid as the test determination. The phosphorus values found

in this way were converted into phospholipids in the form of "lecithin" by multiplying with the factor of 26, and were calculated to micrograms per 100 c.mm. of blood or plasma.

To check the accuracy of the above method, 150 c.mm. of ether solution containing various amounts of cephalin were treated as described above. The results obtained are presented in Table I.

*Plasma Phosphatase Activity in Rats with Obstructive Jaundice*—Jaundice was produced by ligation of the bile duct. Adult rats were anesthetized with sodium amytal and ether. A median incision was begun about 1 cm. anterior to the xiphoid and extended down 3 to 4 cm. The median and the left lobes of the liver were held back with blunt forceps to expose the bile duct. A curved needle threaded with silk was introduced under the duct about 1 cm. from the point where it joined the duodenum and tied.

TABLE I  
*Recovery of Cephalin Phosphorus by Ultramicromethod*

P added, $\gamma$					
0.2	0.3	0.4	0.5	0.6	0.7
P found, $\gamma$					
0.20	0.32	0.44	0.50	0.60	0.68
0.18	0.30	0.40	0.50	0.58	0.72
0.20	0.32	0.42	0.48	0.58	0.70
0.20	0.28	0.40	0.48	0.58	0.66
Average 0.195	0.30	0.415	0.49	0.585	0.69
% recovery 97.5	100.0	103.0	98.0	97.5	98.5

The incision was closed with two layers of silk sutures. In the control operations the silk was simply passed under the bile duct without ligating it.

The rats with ligated bile ducts developed jaundice during the 1st day as indicated by the yellow color of the urine, plasma, paws, and ears. In most cases, however, after intervals of 4 to 8 days visible, new bile ducts were formed and the symptoms of jaundice gradually disappeared, although postmortem examination showed the original duct to be greatly distended. The formation of new bile ducts in rats was demonstrated by histological studies of the liver, and was also manifested in the return to the normal level of the plasma phosphatase activity and phospholipid content of the blood.

Changes in plasma phosphatase activity during jaundice have been widely investigated. In most cases of obstructive jaundice an increase in the plasma phosphatase activity was noted (5-11); in cats, however, no

significant changes were observed (12, 13). In our experiments on jaundiced rats fed on Purina dog chow the plasma phosphatase activity dropped markedly as early as 1 day after the operation. The phosphatase level remained low over a period of 6 to 8 days, after which the enzyme activity increased rapidly and rose above the postoperative level and then gradually returned to normal. While the sudden drop in plasma phosphatase activity resulted from obstruction of the bile ducts, the rise which followed was associated with the functioning of new bile ducts, as indicated by the disappearance of jaundice symptoms and by postmortem examinations.

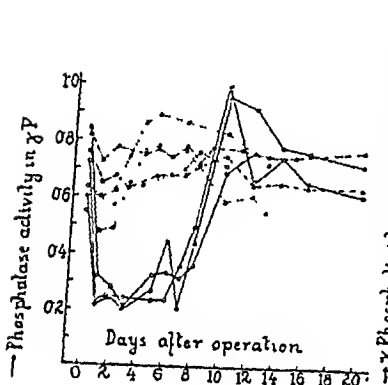


FIG. 1

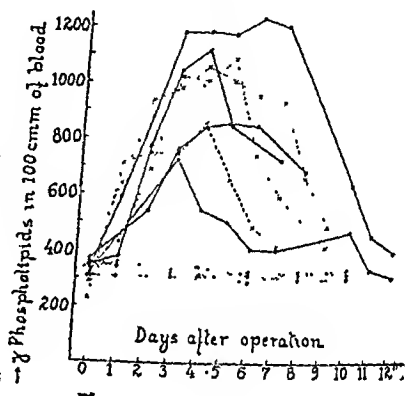


FIG. 2

FIG. 1. Plasma phosphatase activity in rats with obstructive jaundice. ●—● control rats, ○—○ rats with ligated bile ducts fed on Purina dog chow, ○—○ rats with ligated bile ducts fed on Purina dog chow plus ox bile.

FIG. 2. Blood phospholipids in rats with obstructive jaundice. ○—○ control rats, ○—○ rats with ligated bile ducts fed on Purina dog chow, ×—× rats with ligated bile ducts fed on Purina dog chow plus ox bile.

Fig. 1 presents the results obtained and shows that the control operation did not produce any significant changes in the plasma phosphatase activity.

When 3 per cent of ox bile (Eli Lilly and Company, powdered extract No. 88) was added to the Purina dog chow diet and given to rats with ligated bile ducts, only a very slight drop in plasma phosphatase activity was observed. Since control operations on rats produced similar effects, the observed slight decrease can be attributed to secondary influences. After the 3rd day a marked rise in the enzyme activity occurred. The symptoms of severe jaundice persisted for a period of 6 days, then decreased. Postmortem examinations showed that the originally ligated bile duct was greatly distended but new bile ducts were formed. The

results obtained are included in Fig. 1. The ox bile preparation used in these experiments showed no phosphatase activity even when tested over a period of 30 hours at 37°.

*Phospholipids of Whole Blood and Plasma in Rats with Ligated Bile Ducts*—The phospholipids of whole blood were greatly increased in rats with ligated bile ducts. This increase, which reached its maximum in 3 to 7 days, was followed by a gradual decrease owing to the new bile duct formation. The results, graphically presented in Fig. 2, show the contrast between the values obtained on control rats, which were practically constant during the experimental period, and those observed on the rats with ligated ducts. Chanutin and Ludewig (14) also observed an increase of phospholipids in rats with obstructive jaundice.

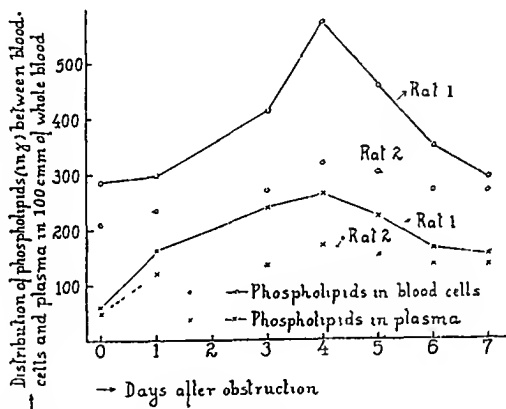


FIG. 3. Distribution of phospholipids between blood cells and plasma in 100 c mm. of whole blood.

The addition of 3 per cent ox bile to the Purina dog chow fed to rats with ligated bile ducts did not prevent the rise in blood phospholipids, as is shown in Fig. 2.

Fig. 3 shows the distribution of phospholipids between blood cells and plasma. For the determination of volume ratio of corpuscles and plasma, the blood was centrifuged in hematocrit tubes at 3000 R.P.M. until no change in volume was observed. Phospholipid was determined directly in whole blood and plasma and the content of the corpuscles was calculated. Fig. 3 shows that the increase of phospholipids in the plasma is relatively higher than that of the blood cells, and also that the absolute value of phospholipids in blood cells is much higher than that of the plasma (15).

Our experiments were extended to include the changes which occur in

the total lipid and phospholipid content of the liver and carcass of rat with obstructive jaundice. The liver and carcass were ground with sand (the carcass having first been passed through a meat grinder) and then extracted with 25 parts of alcohol-ether mixture (3:1). The extraction process was repeated three times for the carcass. The extracts were evaporated in a vacuum, and the residue taken up in chloroform and filtered. The total lipids were estimated by evaporating the chloroform extract and weighing the residue. The phospholipids were estimated by the method of King (16). Table II presents the results obtained on five control rats and on five jaundiced rats. The rats were sacrificed 6 days after the operation. As Table II indicates, no significant differences were observed in the total phospholipids of the liver of normal and jaundiced rats, while a marked decrease in the total lipid content of the carcass was

TABLE II  
*Total Lipid and Phospholipid Content of Liver and Carcass of Normal and Jaundiced Rats*

The results are expressed in per cent of wet tissue

Rat No.	Normal rats				Rat No	Jaundiced rats			
	Phospholipids		Total lipids			Phospholipids		Total lipids	
	Liver	Carcass	Liver	Carcass		Liver	Carcass	Liver	Carcass
1	2 60	1 15	5.99	8 95	6	2 81	1 18	4 44	4 63
2	2 80	1 04	5 55	7 50	7	2 83	1 01	4 96	5 05
3	2 61	1 32	3 87	7 68	8	3 50	1 10	5 58	4 63
4	3.21	1 26	4 17	8 02	9	2 71	1 06	4 63	4 12
5	3.23	1 20	6 26	10 95	10	2 54	0 99	5 06	5 24

noted in jaundiced rats. The phospholipid content of the carcass in jaundiced rats did not differ from that of normal rats

#### DISCUSSION

In our previous work it had been observed that the plasma phosphatase activity of rats was closely associated with the ingestion of certain unsaturated fatty acids (1). By preventing the bile from entering the intestinal tract, the resorption of fats is inhibited and as a result the plasma phosphatase activity decreases sharply. Ox bile added to the diet corrects this defect in resorption and practically eliminates the decrease in enzyme activity of the plasma. The fact that the plasma phosphatase activity does not decrease in rats with ligated bile duct on addition of ox bile to the diet, although the ox bile itself does not have this enzyme activity, would indicate that the phosphatase is not of biliary origin, but



comes from other sources when resorption is restored. These findings would also indicate that the sharp drop in the plasma phosphatase activity in jaundiced rats is not due to the inhibiting action of bile substances in the blood (17), since jaundiced rats fed on a diet containing ox bile developed all the symptoms of severe jaundice without a lowering of the plasma phosphatase activity. The marked increase in plasma phosphatase activity observed in most cases of obstructive jaundice in human patients and in dogs (5-11) supports this assumption.

A remarkably sharp increase was found in the blood phospholipids in rats with ligated bile ducts. This rise was observed both in the blood cells and plasma and was not corrected by the addition of ox bile to the diet. The difference in response of plasma phosphatase and blood phospholipids of jaundiced rats to a diet containing ox bile, however, requires further investigation.

Since fat resorption in the intestinal tract is impaired by the ligation of the bile duct, the great increase of blood phospholipids must be of internal origin. While no changes were observed in the total lipid and phospholipid content of the liver of normal and jaundiced rats, the total lipid content of the carcass of the jaundiced rats was markedly lower than that of the normal animals. It would appear that in the case of jaundiced rats in which the normal fat resorption is prevented the body mobilizes the depot fat and converts it into phospholipids. The marked accumulation of these compounds in the blood (which serves as a transport agent) may be due to that mobilization and partly to the faulty fat metabolism of the diseased animal.<sup>1</sup> The phospholipid content of the carcass was practically the same in both cases and may represent the essential components of the tissue cells (18).

#### SUMMARY

A marked decrease in the plasma phosphatase activity was observed in rats with ligated bile ducts. The subsequent return to normal was associated with new bile duct formation.

The drop in plasma phosphatase activity in jaundiced rats was avoided when 3 per cent ox bile was added to the diet.

An ultramicromethod for the determination of blood phospholipids was developed.

A sharp increase in blood phospholipids was observed in jaundiced rats, followed by a gradual decline toward the normal as new bile ducts were formed. The increase in blood phospholipids in jaundiced animals was not eliminated by the addition of ox bile to the diet.

The increase of blood phospholipids in the jaundiced rat occurs both in

<sup>1</sup> Greig, M. E., and Munro, M. P., private communication.

the blood cells and the plasma. The relative increase in the plasma was higher, although the absolute phospholipid content of the blood cells was much greater than that of the plasma.

No significant difference was observed between the total lipid and phospholipid content of the liver of normal and of jaundiced rats. A marked drop in the total lipid content of the carcass of the jaundiced rat was found. The phospholipid concentration of the carcass of the normal and jaundiced rat showed no significant difference.

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# THE FRUIT OF PYRACANTHA ANGUSTIFOLIA: A PRACTICAL SOURCE OF PRO- $\gamma$ -CAROTENE AND PROLYCOPENE

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While the generally known and wide-spread  $C_{40}$  carotenoids presumably possess an all-*trans* configuration, two representatives of a stereochemically new class of naturally occurring polyenes were recently described (1, 2). Of the seven and six double bonds sterically available for *trans-cis* shifts, prolycopene and pro- $\gamma$ -carotene contain 5-6 and 4-5 *cis* double bonds respectively. On addition of iodine to solutions of these compounds, the corresponding all-*trans* isomer is formed in each case together with minor stereoisomers. This becomes evident by an instantaneous shift in the spectra. After catalysis, the first maxima are located at  $34\mu$  and  $32\mu$  longer wave-length respectively than those of the original solutions.<sup>1</sup>

Prolycopene,  $C_{40}H_{56}$ , was first isolated from the tangerine tomato (a variety of *Lycopersicum esculentum*) in a yield of 20.6 mg. per kilo of fresh material (3). Much less satisfactory was the yield of pro- $\gamma$ -carotene,  $C_{40}H_{56}$ ; viz., 0.3 mg. per kilo of fresh palm fruit (*Butia capitata* Becc.) (4). In the interests of further stereochemical work, a great variety of plant material was tested for "pro" carotenoids in our laboratory by means of extraction, chromatography, and iodine catalysis.

As a result of these analyses, it has been found that the ripe fruit of *Pyracantha* (*Cotoneaster*) *angustifolia* Schneid. (Pomoideae) constitutes the only practical source of pro- $\gamma$ -carotene at the present time; 27.7 mg. were obtained in crystalline form from 1 kilo of air-dried berries (about 3 kilos of fresh material). The same quantity also yielded 28.4 mg. of prolycopene. Furthermore, a second member of the stereoisomeric series, lycopene-prolycopene, was isolated (7.3 mg. of crystals) but we cannot claim with certainty that it is a natural product.

Since all the pigments mentioned are hydrocarbons, the question arises whether the occurrence of "pro" compounds in the vegetable kingdom is restricted to this type. A minor constituent of the *Pyracantha* pigment gave information on this point. Since its spectrum is identical with that of pro- $\gamma$ -carotene before and after the addition of iodine, both must

\* Contribution No. 880.

<sup>1</sup> All spectral data refer to solutions in petroleum ether (b.p. 60-70°) unless otherwise indicated.

possess a similar chromophore. On the other hand, the behavior of the compound in the partition test and especially its increased adsorption affinity as compared with pro- $\gamma$ -carotene (and even  $\gamma$ -carotene) prove the presence of a hydroxyl group. Because of the small quantities available, this monohydroxy pro- $\gamma$ -carotene has not yet been prepared in crystalline form.

It should be noted that all "pro" compounds known at the present time possess at least one aliphatic end-group in their molecules.

We wish to thank Dr. W. Hertrich, Curator of the Huntington Botanical Garden, for his courtesy, and Dr. G. Oppenheimer as well as Mr. G. Swinehart for the microanalyses.

#### EXPERIMENTAL

The *Pyracantha* berries were picked in November and December in Southern California and dried in air at room temperature. On prolonged standing the yields diminish rapidly. 1 kilo of the air-dried material was coarsely ground in a mill, kept under peroxide-free ether for 3 hours, then filtered on a Buchner funnel, washed with ether, and treated once more in the same manner. The extract (4.5 liters) was saponified for a day by keeping it over concentrated methanolic potassium hydroxide. After addition of water the ether solution was washed alkali-free. (The dark wash water did not contain carotenoids.) The solution was dried rapidly with sodium sulfate and evaporated. In order to remove small quantities of ether which would disturb the subsequent adsorption, some petroleum ether was added and the evaporation repeated.

The solution of the dark red, viscous residue in 1 liter of petroleum ether was chromatographed in a large percolator (45  $\times$  20  $\times$  8 cm.) on calcium hydroxide (Shell brand lime, chemical hydrate, 98 per cent passing through a 325 mesh screen). The chromatogram was developed with 5 liters of petroleum ether and then with the same solvent containing 1 per cent acetone. The complicated chromatogram was composed of three sections: (a) a strongly adsorbed, poorly differentiated top section (7 cm. wide), (b) a main section (6 cm.) containing several orange and yellow zones including polycopene and pro- $\gamma$ -carotene, and (c) the lowest section of the cone, occupied by large amounts of  $\beta$ -carotene preceded and followed by some of its stereoisomers. This last section and the yellow, fluorescing filtrate were discarded.

The percolator was inverted and the cone removed in one piece by tapping on the glass with the palm of the hand; the three sections were separated by cutting.

*Fractionation of Main Section*—After elution with alcohol the pigments

were transferred to petroleum ether and developed on a calcium hydroxide column ( $28 \times 7$  cm.) with petroleum ether containing 2 per cent acetone. The chromatogram had the following appearance (the figures on the left denote the width of the zones).

80 mm.	several minor layers near top
35 "	bright orange, prolycopene (470.5, 441 $m\mu$ )
5 "	orange, traces
12 "	yellow, unidentified Pigment 1 (464, 438 $m\mu$ )
10 "	several minor layers
50 "	orange, pro- $\gamma$ -carotene (462, 432.5 $m\mu$ )
10 "	yellow, unidentified Pigment 2 (457.5, 430.5 $m\mu$ )
20 "	several minor layers

The presence of pro- $\gamma$ -carotene and of prolycopene was detected by the addition of iodine to the respective solutions in a spectroscopic cell. By this catalysis intense new spectra appeared almost instantaneously (493, 460  $m\mu$  and 501.5, 470  $m\mu$ ).

*Pro- $\gamma$ -carotene*—This zone was rechromatographed and developed with petroleum ether containing 2 per cent acetone; the main component was eluted with ether. Upon evaporation of the ether *in vacuo* a dark red, crystalline residue remained. The latter was dissolved in the minimum amount of benzene at  $20^\circ$  and transferred into a 15 cc. centrifuge tube with a dropper. About 10 cc. of methanol were then added with stirring, first drop by drop until red crystals appeared, and later more rapidly. After standing in ice water for  $\frac{1}{2}$  hour, the crystals were centrifuged and washed with methanol in the same tube. After recrystallization from benzene and methanol, the yield was 25.1 mg. The mother liquor gave 2.6 mg. The total yield corresponds to 45 per cent of the pro- $\gamma$ -carotene content of *Pyraacantha* as estimated photometrically. M.p.,  $121$ – $122^\circ$  (corrected) (after softening near  $119^\circ$ ; the sealed tube was filled with  $\text{CO}_2$ ; the sample was introduced into the Berl block  $20^\circ$  below the melting point). The crystal form has been described (4). The partition behavior (petroleum ether-95 per cent methanol) was epiphasic. A mixed chromatogram with pro- $\gamma$ -carotene from *Butia capitata* established the identity of the two samples. For the purpose of analysis the substance was dried at  $50^\circ$  in a high vacuum for 45 minutes. It was free of ash.

*Analysis*— $\text{C}_{40}\text{H}_{56}$ . Calculated. C 89.48, H 10.52  
 Found. " 89.79, 89.94, " 10.55, 10.52  
 Mol. wt. (in exaltone), calculated, 537; found, 558

The absorption maxima in carbon disulfide were 492.5, 459  $m\mu$  (after the addition of iodine, 529.5, 491.5  $m\mu$ ); in benzene, 475.5, 446.5  $m\mu$  (with iodine, 506.5, 472  $m\mu$ ); and in petroleum ether, 462, 432.5  $m\mu$ , (with iodine,

493, 460  $m\mu$ ). In all cases a slight shadow was observed at about 30  $m\mu$  longer wave-length than the first maximum.

*Polycopene*—This layer was cut out and rechromatographed. By developing with petroleum ether containing 5 per cent acetone, minor layers were separated. The main pigment was then eluted and isolated as described for pro- $\gamma$ -carotene. A recrystallization was not carried out in this case. The yield was 28.4 mg.; i.e., about 40 per cent of the quantity contained in the original extract as estimated photometrically. M.p., 110.5–111.5° (corrected). The shape of the crystals and their solubility corresponded with those of a sample from tangerine tomatoes. A mixed chromatogram showed no separation. In the partition test (petroleum ether-95 per cent methanol) epiphasic behavior was observed. For the purpose of analysis the sample was dried in a high vacuum at room temperature for 45 minutes.

*Analysis*— $C_{40}H_{56}$

Calculated. C 89.48, H 10.52

Found. " 89.39, " 10.63 (corrected for 0.7 % ash)

Mol. wt. (in exaltone), calculated, 537; found, 575

The absorption maxima in carbon disulfide were 500, 468  $m\mu$  (with iodine, 512.5, 502.5, 468.5  $m\mu$ ); in benzene, 482.5, 453  $m\mu$  (with iodine, 518.5, 483, 452.5  $m\mu$ ); and in petroleum ether, 470.5, 441  $m\mu$  (with iodine, 501.5, 469.5, 439.5  $m\mu$ ).

*Minor Pigments*—The unidentified Pigment 1, after having been rechromatographed, showed absorption maxima at 464.5, 435.5  $m\mu$  which were shifted on iodine catalysis to 469, 439  $m\mu$ . This equilibrium mixture when chromatographed and developed with petroleum ether containing 5 per cent acetone separated into five layers; the spectrum of the main pigment, adsorbed near the top, was 471, 442  $m\mu$ .

The unidentified Pigment 2, after having been rechromatographed and treated with iodine, separated upon chromatographing into two isomers (458, 431  $m\mu$  and 454, 426  $m\mu$  respectively, from the top of the column) which gave identical spectra (457, 429  $m\mu$ ) on treatment with iodine.

*Fractionation of Top Section*—The pigments were eluted with alcohol, transferred to petroleum ether, and developed on calcium hydroxide in a smaller percolator (30  $\times$  11  $\times$  6 cm.) with the solvent mentioned containing 5 per cent acetone. Five main fractions (Fractions I to V from top to bottom) appeared, each consisting of several pigments.

Fraction I (which among other pigments contained some lutein) and Fraction III were of no particular interest.

Fraction II, when rechromatographed and developed with 10 per cent acetone in petroleum ether, separated into nine components; viz., lycopenes, two neolycopenes, a monohydroxy pro- $\gamma$ -carotene, and five minor layers.

The monohydroxy pro- $\gamma$ -carotene zone was rechromatographed and was then homogeneous. The main absorption maxima were 461.5, 432.5  $m\mu$  and on iodine catalysis, 492.5, 459  $m\mu$ . In contrast to pro- $\gamma$ -carotene this pigment was present in both phases if a drop of water was added to the solution in a petroleum ether-methanol mixture. When a petroleum ether solution was shaken with 90 per cent methanol, epiphasic behavior was observed. The adsorbability is also conclusive evidence for the presence of a hydroxyl group. On calcium hydroxide the compound is adsorbed below lycopene but much above pro- $\gamma$ -carotene, as shown by mixed chromatograms. Furthermore, after the addition of iodine a main component is formed which does not separate on the column from a monohydroxy- $\gamma$ -carotene (probably rubixanthin) obtained from another source. Finally, monohydroxy pro- $\gamma$ -carotene is also adsorbed on calcium carbonate from petroleum ether, which, as is well known, does not occur with hydrocarbon carotenoids.

Fraction IV consisted mainly of an orange pigment which showed maxima at 474, 442.5  $m\mu$  (with iodine, 501.5, 469.5, 439  $m\mu$ ). In carbon disulfide the corresponding figures were 504, 471.5  $m\mu$  and 543, 502.5, 468  $m\mu$ . This stereoisomer of lycopene was eluted with ether and crystallized as described above for pro- $\gamma$ -carotene. The yield was 7.3 mg.; m.p., 97–98° (corrected) (after softening). The main pigment formed by iodine catalysis did not separate from tomato lycopene in a mixed chromatogram.

Analysis— $C_{40}H_{56}$ .	Calculated.	Mol. wt. 537
	Found.	" " 563 (in caltane)

Fraction V, when rechromatographed on calcium hydroxide with 25 per cent ligroin in benzene, separated into several minor carotenoids and two main pigments with  $\gamma$ -carotene spectra (495, 462  $m\mu$  and 494, 461  $m\mu$ ). On addition of iodine maxima of shorter wave-length appeared. Both have been crystallized and will be investigated. As remarked earlier (4) some observations seem to point to the existence of two  $\gamma$ -carotenes, which possibly differ only in the position of the isolated double bond.

#### SUMMARY

Ripe fruit of *Pyracantha* (*Cotoneaster*) *angustifolia* Schneid constitutes the best practical source for the isolation of pro- $\gamma$ -carotene,  $C_{40}H_{56}$ , and a good source for prolycopene,  $C_{40}H_{56}$ , both of which possess partially *cis* configurations. The yields were 27.7 mg. of crystallized pro- $\gamma$ -carotene and 28.4 mg of prolycopene from 1 kilo of air-dried berries. A close stereoisomer of prolycopene was also isolated (7.3 mg) and a monohydroxy pro- $\gamma$ -carotene observed in solution.

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# ISOLATION OF POLYCOPENE AND PRO- $\gamma$ -CAROTENE FROM EVONYMUS FORTUNEI

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It has been reported (1) that the ripe seeds of *Evonymus europaeus* L. (Celastraceae) contain unesterified zeaxanthin,  $C_{40}H_{56}O_2$ , as the main polyene pigment. The same statement is valid for *Evonymus fortunei*, var. color., Rehd., commonly termed "winter-creeper euonymus," from 1 kilo of which 1300 mg. of zeaxanthin were isolated. Upon evaporation of its saponified ether extract and addition of petroleum ether, abundant quantities of zeaxanthin crystallize. This paper describes an investigation of some of the pigments (about 200 mg. per kilo, of which one-fourth is  $\beta$ -carotene) which remain in the mother liquor. The mixture can be resolved by chromatographic analysis. In addition to some twenty less interesting pigments, two representatives of a stereochemically new class of natural carotenoids (2) were separated; viz., polycopene,  $C_{40}H_{56}$ , and pro- $\gamma$ -carotene,  $C_{40}H_{56}$ . The yields of pure crystals were 11 mg. and 0.5 mg. per kilo of seeds respectively. Hence, *Evonymus fortunei* may serve as a source of polycopene while it does not offer any larger yield of pro- $\gamma$ -carotene than does the fruit of *Butia capitata* (3). According to the foregoing paper (4) *Pyracantha angustifolia* is the best starting material for the isolation of pro- $\gamma$ -carotene at the present time.

We wish to thank Mr. F. W. Westcourt of the Texas State College for Women for the identification of the plant, and Dr. G. Oppenheimer and Mr. G. Swinehart for a microanalysis.

## EXPERIMENTAL

The material was collected in Denton, Texas. The intensely orange-red hulls of 1 kilo of seeds were scraped off by rubbing between two layers of wire gauze in a mortar. Small particles of hull remained on the stones and were neglected. The pigment- and lipid-rich hulls were ground with sand and extracted with peroxide-free ether by repeated shaking. The dark extract (2 liters) was saponified over concentrated methanolic potassium hydroxide for 20 hours, then washed alkali-free, dried with sodium sulfate, and evaporated *in vacuo* at 40° as far as possible. To the dark, partially

\* Contribution No. 881.

crystalline residue, petroleum ether (b.p. 60–70°) was repeatedly added and evaporated. Finally, the oily residue was dissolved in the minimum volume of chloroform. On addition of 3 volumes of petroleum ether the main bulk of zeaxanthin crystallized out.

The mother liquor was poured on calcium hydroxide (Shell brand lime, chemical hydrate, 98 per cent passing through a 325 mesh screen) in a percolator (45  $\times$  20  $\times$  8 cm.). After washing the chloroform from the adsorbent with petroleum ether, the chromatogram was developed in the course of 3 hours with petroleum ether containing 2 per cent and later 3 per cent acetone. The light orange filtrate was discarded; it contained among other polyenes a portion of the  $\beta$ -carotene.

The cone was cut into three parts. The upper section (160 mm. from the top) was composed of an orange-brown (20 mm.) and a red (140 mm.) part, both of which were heterogeneous. Then followed an orange section (100 mm.) containing the two "pro" compounds and some minor pigments. In the lowest section  $\beta$ -carotene predominated (60 mg.). The middle section was eluted with alcohol, transferred to petroleum ether, and developed on a calcium hydroxide column (28  $\times$  7 cm.) with 1 liter of petroleum ether containing 2.5 per cent acetone and then with 0.5 liter containing 4 per cent. The following chromatogram appeared (on the left side the height of the zones is given).

55 mm.,	pink
40 "	orange, contained prolycopene
4 "	greenish yellow
3 "	light orange
5 "	yellow
20 "	orange, contained pro- $\gamma$ -carotene
7 "	greenish yellow
15 "	faint orange
0.5 "	green line
15 "	colorless
7 "	pink, $\beta$ -carotene

*Prolycopene*.—This zone was cut out, eluted with ether, dried, and evaporated *in vacuo*. The residue was dissolved in the minimum amount of benzene and crystallized in a centrifuge tube by cautious addition of several volumes of methanol. The microscope showed typical prolycopene crystals intermixed with much colorless crystalline material. The latter could not be removed by recrystallization from benzene and methanol and only partially by treatment with methanol at 40°. It was almost completely removed by short centrifuging at slow speed. The heavy pigment crystals settled and the suspended colorless compound was decanted. Minor amounts of prolycopene in the decanted liquid were recovered by repeating the process. The last trace of the contaminant was removed

by a short treatment with methanol at  $40^{\circ}$  and rapid centrifuging. The yield was 11 mg.; m.p.,  $109-110^{\circ}$  (corrected; in a sealed tube filled with  $\text{CO}_2$ ). For the purpose of analysis the sample was dried at about  $45^{\circ}$  in a high vacuum for 45 minutes; it was free of ash.

Analysis— $\text{C}_{40}\text{H}_{56}$ . Calculated. C 89.48, H 10.52

Found. "89.00, " 10.72

Mol. wt., calculated, 537; found, 529 (in exaltone)

In a mixed chromatogram the pigment did not separate from prolycopene obtained from tangerine tomatoes (2). The spectral maxima of fresh solutions were in carbon disulfide 500.5, 469.5  $\text{m}\mu$  (after the addition of iodine, 540.5, 500.5, 466  $\text{m}\mu$ ) and in petroleum ether 470, 442  $\text{m}\mu$  (with iodine, 501, 469, 441  $\text{m}\mu$ ).

*Pro- $\gamma$ -carotene*—This zone of the above chromatogram was eluted with alcohol, transferred into 20 cc. of petroleum ether, and rechromatographed on a calcium hydroxide column ( $27 \times 5$  cm.). Minor layers located both above and below the main orange pigment were discarded and the latter was rechromatographed on a smaller column ( $20 \times 3$  cm.) of the same adsorbent. This showed only traces of other pigments, much above pro- $\gamma$ -carotene. The latter was eluted with ether and the evaporation residue was crystallized from benzene and methanol as described for prolycopene. No colorless contaminant was present. The yield was 0.5 mg.; the crystal form was typical for pro- $\gamma$ -carotene. In a mixed chromatogram no separation took place from a sample isolated from *Pyracantha angustifolia* (4). Spectral maxima in carbon disulfide were 492.5, 459  $\text{m}\mu$  (with iodine, 527.5, 490  $\text{m}\mu$ ) and in petroleum ether, 461.5, 431.5  $\text{m}\mu$  (with iodine, 490, 457.5  $\text{m}\mu$ ).

#### SUMMARY

From 1 kilo of the ripe seeds of *Evonymus fortunei* Rehd., 11 mg. of prolycopene,  $\text{C}_{40}\text{H}_{56}$ , and 0.5 mg. of pro- $\gamma$ -carotene,  $\text{C}_{40}\text{H}_{56}$ , have been obtained in crystalline form.

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# ARTERIAL AND CEREBRAL VENOUS BLOOD

## ARTERIAL-VENOUS DIFFERENCES IN MAN\*

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The immediate purpose of this study is the determination of the norm for certain chemical constituents of the arterial and internal jugular blood of healthy young men. It is obvious that if a complete balance sheet can be made out, showing what is taken from and what is added to the blood in its passage through the brain, much light will be shed on the problems of brain metabolism. No complete balance sheet can be submitted as a result of the present study but presentation of a partial list of blood constituents is a considerable addition to the limited amount of data available on the metabolism of the normal human brain. The brains of the subjects examined were not only structurally normal as evidenced by the lack of abnormal neurological signs or symptoms, but were functionally normal as shown by electroencephalographic examinations. Arterial and venous samples were drawn simultaneously, so that arterial-venous differences could be determined and an accurate estimate made of the changes that occur in the blood as it flows through the brain.

### *Methods*

50 males, 18 to 29 years of age, almost all of them medical students, were chosen for examination. The subjects lay quietly for at least  $\frac{1}{2}$  hour before blood was drawn from the internal jugular vein and the femoral, radial, or brachial artery. The internal jugular vein was punctured by the method described by Myerson, Halloran, and Hirsch (10). Care was taken to see that the subjects breathed normally during and immediately preceding the taking of the sample.

Blood was taken without exposure to air and gas determinations were made as soon as possible on samples of whole blood, to which potassium oxalate was added as an anticoagulant. The Van Slyke manometric method of blood gas analysis was employed and the procedures recommended by Peters and Van Slyke (11, b) followed. The pH of whole blood was determined immediately at 38° electrometrically. A glass

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electrode amplifier system was used which permitted pH measurements to be made with a precision of  $\pm 0.002$  pH unit. The pH values recorded are in accordance with the pH scale recommended by Hitchcock and Taylor (7). Lactic acid was also determined on whole blood, to which oxalate and fluoride had been added according to the method proposed by Edwards (3). The procedures devised by Folin and Wu (4) were used for the determination of sugar. The total base of the serum was determined by electrodialysis, and the modifications proposed by Consolazio and Talbott (2) were followed. Inorganic phosphorus was measured by the procedures of Kuttner and Lichtenstein (8).

### Results

The analytical results for each of the 50 subjects and the calculated carbon dioxide tensions for the samples of their bloods are presented in Table I. The method by which the carbon dioxide tension was calculated is given in Peters and Van Slyke ((11, a) pp. 912-913). The various arterial-venous differences which can be obtained from Table I are particularly important, since these values give the pertinent information concerning the chemical changes that take place in the brain. Table II contains the means, extremes, and standard deviations of all the blood constituents investigated and their arterial-venous differences.

*Oxygen*—The metabolic demands of the human brain are such that under conditions of average blood flow the oxygen content of the blood passing through the brain is reduced by 6.7 volumes per cent. The blood leaves the brain with a saturation of 61.8 per cent, a net reduction in saturation of 32.1 per cent. The loss of oxygen from the blood to the brain is somewhat larger than the average loss of oxygen to the tissues throughout the rest of the relaxed body. Therefore, from a consideration of the blood supply to the brain it would seem that the *in vivo* metabolism of this tissue is well above the average metabolism of the body as a whole.

The average oxygen capacity of arterial blood and whole blood from the internal jugular vein (Table II) is identical within the experimental error. In some individuals (Table I) there may be a small difference, indicating that the blood has become slightly diluted as it passes through the brain.

*pH*—The average change in acidity of the blood as it flows through the brain is 0.053 pH unit (Table II). This change is somewhat larger than the 0.02 to 0.03 unit obtained by Bock *et al.* (1) for blood passing through resting muscle, which again indicates a greater chemical activity in the brain. The pH values of the arterial and venous blood in themselves are of interest. The arterial blood of the young men in this series had an average value of 7.424 pH units and the venous blood an average value of 7.371 units. The mean of the two values is 7.396 units. Shock and

Hastings (12), using colorimetric methods, found that finger blood from males had an average value of 7.399 units. A comparison of all of the various measurements of finger blood by these authors indicates that, although finger blood bears a closer resemblance to arterial than to venous blood, the individual samples are more variable. The range for the various components of the acid-base balance of finger blood includes practically all of the values reported here for the arterial and the internal jugular blood.

*Carbon Dioxide*—Blood as it flows through the brain gains on the average 6.6 volumes per cent of carbon dioxide (Table II). From the data on oxygen and pH it is possible to calculate that the increase in carbon dioxide tension produced by this increment of carbon dioxide is 9.97 mm. of mercury. Because of the rapid diffusibility of carbon dioxide through the tissues, the tension of carbon dioxide in the brain should be only slightly greater than its tension in the internal jugular vein; the latter value was 49.9 mm. of mercury.

The average carbon dioxide content of arterial blood was 21.6 mm per liter (48.2 volumes per cent) and of the venous blood 24.6 mm (54.8 volumes per cent) (Table II). The mean of these two, 23.1 mm, is slightly above the average value for finger blood, 22.3 mm, reported by Shock and Hastings (12).

In this group of 50 normal young men at rest, the carbon dioxide content of arterial blood never exceeded 50.5 volumes per cent (Table II). Out of a total series of 108 normal men studied in our laboratory only one had an arterial carbon dioxide content above 50.5. In a group of 59 grand mal epileptics examined by Gibbs, Lennox, and Gibbs (5), the carbon dioxide content was above this level in 61 per cent, and it was found that patients affected only with petit mal epilepsy tend to have a low arterial carbon dioxide content. From these and other studies, it would seem that epileptics have some disturbances in the mechanisms which govern their acid-base balance.

*Respiratory Quotient*—Himwich and Nahum (6) demonstrated that the respiratory quotient of cat brain *in vivo* is close to 1, and Lennox and Leonhardt (9) obtained a value close to 1 for the human brain. The present results (Table I) serve to attest the accuracy of these earlier findings, for the average respiratory quotient for our 50 subjects was 0.99.

*Sugar and Lactic Acid*—Under conditions of average blood flow 10 mg. of sugar are removed from every 100 cc. of blood passing through the brain (Table II). If this sugar is all dextrose and completely oxidized, it would require 7.46 cc. of oxygen per 100 cc. of blood. But some lactic acid is produced; the average value for lactic acid contributed by the brain is 1.6 mg. per 100 cc. of blood (Table II). If this lactic acid were





21	24	18.8	13.2	20.1	20.1	93.6	65.9	7.415	7.373	47.2	52.8	39.3	47.3	153.0	154.4	2.8	2.8	7.5	8.6	96	87
22	23	18.8	12.5	20.4	20.4	92.5	61.3	7.417	7.357	44.6	51.0	37.3	47.4	151.0	152.0	3.4	3.4	15.2	15.7	92	84
23	24	20.9	12.5	22.4	22.4	93.3	56.0	7.455	7.381	47.3	55.4	37.5	50.0	152.6	152.8	3.7	3.7	9.9	10.5	82	71
24	22	20.0	13.9	21.2	21.0	93.8	66.0	7.435	7.376	49.4	54.9	40.2	49.5	150.0	150.6	3.3	3.3	7.7	8.5	81	70
25	29	19.4	11.9	20.6	20.3	94.4	58.8	7.425	7.362	49.4	56.8	40.7	52.3	151.8	153.0	3.1	3.1	8.0	8.4	83	74
26	21	20.4	14.2	21.6	21.5	94.2	66.0	7.430	7.373	46.8	53.0	38.5	48.2	152.6	154.0	3.4	3.4	11.1	13.3	102	92
27	19	20.1	13.3	21.9	21.9	91.8	60.5	7.446	7.391	49.1	56.2	39.3	49.6	153.2	154.0	3.1	3.1	9.3	11.4	100	88
28	18	20.1	12.6	21.1	20.8	95.0	60.6	7.405	7.336	46.4	53.9	39.9	52.7	152.2	153.8	3.4	3.4	7.3	9.9	103	95
29	19	19.0	14.5	20.9	20.9	91.3	69.4	7.409	7.375	47.9	52.3	40.8	47.4	151.6	153.2	3.4	3.4	10.2	11.1	96	87
30	20	18.6	11.9	20.1	20.1	92.6	59.3	7.402	7.363	49.3	55.8	42.2	51.2	150.7	151.0	3.1	3.1	7.2	9.6	106	97
31	21	19.4	12.6	20.8	20.9	93.5	60.4	7.386	7.345	45.2	52.1	40.4	49.8	153.0	155.2	3.4	3.4	10.1	12.5	93	82
32	21	19.8	12.1	21.3	21.1	93.2	57.6	7.403	7.346	47.6	55.5	41.3	53.2	155.3	157.8	3.1	3.1	14.0	14.4		
33	23	22.2	15.2	23.4	23.4	94.8	64.9	7.421	7.363	48.2	55.3	41.4	52.5	153.6	154.3	4.0	4.0	8.3	9.2		
34	23	20.9	14.4	22.8	22.8	91.2	63.1	7.429	7.375	47.9	54.4	40.1	50.1	156.2	157.9	4.2	4.2	7.8	8.9		
35	23	20.0	14.2	21.3	21.1	94.0	67.2	7.423	7.363	50.0	55.5	41.6	51.1			3.0	3.0	11.5	13.8		
36	24	20.5	13.1	21.2	21.2	94.3	61.7	7.423	7.366	48.2	55.4	40.2	50.9			3.8	3.8	9.0	9.4		
37	19	18.9	12.9	20.0	19.9	94.6	64.8	7.410	7.353	46.3	52.4	39.1	49.1			3.6	3.6	10.1	11.3		
38	24	18.5	12.0	19.8	19.7	93.5	61.0	7.422	7.372	49.9	56.5	41.0	50.7			4.1	4.1	9.3	13.1		
39	27	19.0	12.3	20.1	20.1	94.2	61.2	7.443	7.397	48.8	56.2	38.5	48.0	150.9	151.6	3.1	3.1	9.0	9.5	96	85
40	24	18.0	11.0	18.7	18.7	95.8	59.1	7.439	7.371	48.2	55.1	37.8	49.0			2.8	2.8	8.5	10.5		
41	22	20.8	13.7	22.0	22.0	94.2	62.5	7.407	7.358	48.1	55.4	41.6	52.4			3.2	3.2	15.2	16.1		
42	28	17.3	11.9	18.4	18.4	93.7	64.3	7.419	7.371	48.4	54.1	39.4	48.0	148.4	151.1	3.9	3.9	11.6	13.3	89	79
43	25	20.4	12.8	21.6	21.6	94.4	59.3	7.422	7.366	47.5	54.5	39.8	49.8	154.1	154.7	3.2	3.2	8.6	11.0	85	75
44	22	19.7	12.0	20.9	20.8	94.6	57.5	7.421	7.369	48.9	56.3	40.8	51.3	149.8	149.8	3.3	3.3	8.4	10.8	95	85
45	22	20.7	14.4	22.3	22.3	93.0	64.5	7.435	7.384	48.8	54.9	40.2	49.5	151.0	152.2	3.5	3.5	9.0	11.2	84	77
46	22	19.1	11.5	20.3	20.2	94.2	56.8	7.432	7.380	48.2	55.5	38.9	49.0	155.0	155.6	4.0	4.0	19.1	22.0	83	74
47	26	18.7	11.8	19.9	19.7	93.8	59.9	7.431	7.373	50.4	57.2	40.7	51.1	154.2	155.4	3.5	3.5	7.6	9.3	85	72
48	29	18.9	11.2	20.1	19.9	94.0	56.4	7.439	7.374	45.4	53.1	36.2	47.6	150.3	149.0	3.3	3.3	14.8	16.2	89	78
49	22	22.2	16.1	23.9	23.9	92.7	67.3	7.422	7.392	48.6	54.7	41.7	49.3	159.0	161.1	3.0	3.0	11.7	14.1	83	78
50	21	18.6	11.8	19.9	19.9	93.4	59.4	7.429	7.372	47.2	54.3	38.2	48.7	153.8	160.4	3.5	3.5	7.4	9.4	92	83
Average...		19.6	12.9	20.9	20.8	93.9	61.8	7.424	7.371	48.2	54.8	39.9	49.9	152.9	154.1	3.4	3.4	9.9	11.5	92	82

TABLE II  
*Level of Certain Constituents of Arterial and Internal Jugular Blood from Healthy Young Men at Rest*

Constituent	No. of cases	Arterial blood				Internal jugular blood				Arterial-venous difference			
		Low	High	Average	S. D.	Low	High	Average	S. D.	Low	High	Average	S. D.
O <sub>2</sub> content, vol. %	50	17.3	22.3	19.6	1.2	11.0	16.1	12.9	1.3	4.5	8.5	6.7	0.8
" capacity, " %	50	18.4	23.9	20.9	1.3	18.4	23.9	20.8	1.3	0	0.3	0.1	
" saturation, %	50	91.2	95.8	93.9	1.0	55.3	70.7	61.8	3.7	21.9	39.1	31.7	3.9
CO <sub>2</sub> content, vol. %	50	44.6	50.4	48.2	1.4	51.0	57.7	54.8	1.6	4.4	8.3	6.6	0.8
" tension, mm. Hg.	50	36.2	44.9	39.9	1.8	46.9	54.3	49.9	1.9	6.6	12.8	10.0	1.2
pH at 38°	50	7.374	7.455	7.424	0.016	7.321	7.397	7.371	0.015	0.030	0.074	0.053	0.009
Lactic acid, mg. %	50	5.9	19.1	9.9	2.6	7.7	22.0	11.5	2.7	0.3	3.8	1.6	0.9
Sugar, mg. %	33	79	127	92	10	70	119	82	10	5	13	9.8	1.7
Inorganic P, mg % serum	50	2.7	4.5	3.4	0.4	2.7	4.5	3.4	0.4				
Total base, m.eq. per l. serum	44	148.4	159.0	152.9	2.2	149.0	161.1	154.1	2.6	0	6.6	1.2	1.2
CO <sub>2</sub> A - V. difference = R.Q.													
O <sub>2</sub> A - V. difference	50									0.90	1.10	0.99	0.03

derived from the dextrose, less oxygen would be required to account for the sugar consumption; only 6.27 cc. of oxygen would be needed, a value only slightly less than the average value of 6.7 cc. of oxygen which is actually observed (Table II). These data support the thesis that the principal source of energy for brain metabolism is sugar, but that not all of this sugar is actually oxidized, since a small but definite amount reappears in the blood stream as lactic acid.

*Total Base*—The concentration of the total base in the serum of the internal jugular vein is on the average 1.2 millicivalents per liter greater than that in the arterial serum (Table II). For the most part, this is not due to any contribution made by the brain, but represents the effect of the redistribution of water between the blood cells and the plasma as the blood becomes venous. The difference of 1.2 milliequivalents is comparable with the difference of 1 to 2 milliequivalents between arterial and venous blood in the arm, as reported by Bock *et al.* (1).

The average value for total base of 154.1 millicivalents per liter of serum from the internal jugular vein is essentially the same as the value of 153.2 milliequivalents from arm vein serum obtained by Consolazio and Talbott (2) in a series of 52 males.

*Inorganic Phosphorus*—There is no detectable change in the level of inorganic phosphorus of the serum of blood which has passed through the brain (Table II). The average level found, 3.4 mg. per cent (Table II), is in the middle of the range 2 to 5 mg. per cent, which is accepted as being within normal limits for inorganic phosphorus in the healthy adult.

#### SUMMARY

The concentration of oxygen, carbon dioxide, pH, lactic acid, sugar, total base, and inorganic phosphorus has been measured in the arterial and internal jugular blood of 50 intelligent, healthy, electroencephalographically normal, young men. Blood samples from an artery and internal jugular vein were drawn simultaneously, thus permitting estimation of the metabolic activity of the brain. These data furnish normal control data for future studies of the metabolism of brains which are functioning abnormally.

The respiratory quotient of the brain in this series is 0.99. This figure together with data on the concentrations of sugar, lactic acid, and oxygen in the blood entering and leaving the brain indicates that sugar is the principal source of energy for the brain *in vivo*. However, not all of the sugar is completely oxidized, for a small part appears to be converted into lactic acid.

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thanks to the subjects who suffered moderately and gave their blood to make this investigation possible.

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# THE RELATION BETWEEN RED BLOOD CELL DENSITY AND CORPUSCULAR HEMOGLOBIN CONCENTRATION

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This investigation is concerned with determinations of red blood cell density and mean corpuscular hemoglobin concentration in man, and with their bearing on the problem of red cell structure. At first it seemed that both the density and the quantity of hemoglobin in unit volume of red blood cells would be simple to measure, but a detailed investigation brought several unexpected difficulties to light, including inadequacies in some of the standard methods.

The samples of blood were obtained by vein puncture from persons acting as blood donors, and preoperatively and postoperatively from a series of patients. None of the cases showed blood dyscrasia, but no attempt was made to confine the investigations to those with a hemoglobin concentration in the normal range. On the contrary, blood from cases of anemia from hemorrhage and of the comparatively mild postoperative anemia formed about half of the material, since variations in hemoglobin concentration were sought rather than avoided. The blood samples were withdrawn into small bottles containing heparin.

## Methods

*Density*—The heparinized whole blood is centrifuged at 4000 R.P.M. for 2 hours in a large International centrifuge. The plasma is drawn off by suction, and the upper layers of cells together with plasma which always remains near the surface are removed with capillary pipettes and blotting paper. If the removal is thoroughly done, the mass of cells remaining is at least 98 per cent packed, as can be shown by spinning a sample in a high speed hematocrit.

A 0.5 ml. micro pipette ("to contain"), with a ground tip and a constriction in the region of the 0.5 ml. mark, is thoroughly dried and weighed to 0.1 mg. A rubber teat is attached, and the pipette is introduced with the teat compressed into the bottom of the tube containing the packed red cells. It is important to compress the teat before the pipette is dipped into the cell mass, and not after, for if air bubbles are expelled they may not rise through the viscous mass, and, drawn up into the pipette where they may remain undetected because of opacity, may affect the density determinations. The sample of the cell mass should be drawn up no further beyond

the mark than can be helped; this is easy, as the mass is very viscous. The teat is removed, and the volume adjusted to the mark by tapping the tip of the pipette on filter paper; the pipette is then wiped off on the outside with a moist cloth, dried, and weighed without delay.

The pipette with its contents is introduced into a 100 ml. flask, the teat reapplied, and the contents forced out. The pipette is then rinsed with three successive 1 ml. volumes of water, so as to transfer all of the sample to the flask without loss.

The weight of the sample of packed cells being known, and the weight of the same volume of water having been found by a calibration of the pipette, the density of the cells can be calculated. Repeated determinations on the same sample of cells show that the variation in the weight of successive samples does not exceed  $\pm 0.5$  mg.; this means that the density can be determined with an accuracy of  $\pm 0.001$ . When the pipette is filled with water, as in calibrating it, the accuracy is not quite so good, for the water, being so much less viscous than the packed cells, is more difficult to adjust to the mark. I have therefore used the average of ten determinations as the weight of water which the pipette holds. The densities are calculated with water at the same temperature taken as unity.

*Iron Determinations*—Wong's method (1) is modified as follows: The sample of packed cells with the added 3 ml. of water is swirled around in the flask until it is hemolyzed and spread evenly over the bottom and sides. This swirling should take several minutes, for it is most important to obtain homogeneity of the material, which tends to form lumps. 4 ml. of concentrated  $\text{H}_2\text{SO}_4$  (Fe-free) are added drop by drop, and the swirling is continued, so that the final mixture is quite homogeneous. After it has stood for about 2 hours,<sup>1</sup> 4.0 ml. of saturated potassium persulfate are added, again with swirling, and the mixture is allowed to stand for another hour. 50 ml. of water are added, and following this 4 ml. of Fe-free sodium tungstate (Klett Manufacturing Company). After another hour of standing, water is added to the 100 ml. mark, and the contents of the flask are filtered through a medium filter paper. The filtrate should be clear and colorless, and when placed in the colorimeter should read substantially the same 0 as water.<sup>2</sup>

To 10 ml. of the filtrate are added 0.5 ml. of saturated potassium persulfate

<sup>1</sup> I have found that the best results with Wong's iron method are obtained if the sulfuric acid and persulfate are allowed to act for these long times, and that quite irregular results are obtained if Wong's original directions (agitating the mixture for a minute or two, cooling under the tap, etc.) are followed.

<sup>2</sup> The filtrate may show a brown tinge if too little acid or tungstate is used, and may become slightly opalescent if allowed to stand too long before color is developed. In either case it must be discarded.

and 2 ml. of 3 N potassium thiocyanate. The red color develops almost instantly, and is read in a Klett-Summerson colorimeter against water set at 0. After some 10 minutes or so, the color slowly fades.

A standard, in which color is developed at the same time as in the unknown, is made by taking 5 ml. of a standard iron solution containing 0.1 mg. of ferric iron per ml., adding 4 ml. of  $\text{H}_2\text{SO}_4$  and 4 ml. of potassium persulfate, making up to the mark, and developing color in a 10 ml. sample in the same way as in the unknown. Under my working conditions, the reading of the standard does not vary by more than  $\pm 1$  per cent from day to day. A blank is prepared by taking 4 ml. of  $\text{H}_2\text{SO}_4$ , 4 ml. of persulfate, making up to 100 ml. with water, and developing the color in a 10 ml. sample. The reading of the blank is small and constant for the same set of reagents.

The results obtained by this method are reproducible with an accuracy of about  $\pm 2$  per cent, and the known amounts of iron added to the sample of packed cells may be measured with about the same precision. Beer's law is obeyed over the working range.

To convert the result of the iron determination into values for hemoglobin, I have taken the iron content of hemoglobin as 336 mg. per cent (see "Discussion").

*Difficulties in Measuring Density*—At first I tried to measure the density of the cells from the density of whole blood, then of the plasma, and the percentage volume as obtained by a high speed hematocrit, but the final figure was found to be very sensitive to errors in the hematocrit determination, and so the method was abandoned.

I then tried a method described by Linderstrøm-Lang (2) for the determination of density. I found two sources of difficulty with this method. (a) Droplets of packed cells do not come to equilibrium as quickly as do droplets of the standard, for because of the high concentration of lipid in the packed cells the drop is not entirely insoluble in the kerosene and bromobenzene, nor is the lipid entirely insoluble in it. There is accordingly some doubt about the equilibrium position, but as the final observation can be made at the end of a fixed time, e.g. 5 minutes, this is a minor difficulty. (b) Successive droplets from the same sample of packed cells do not always take up the same equilibrium position, and the variations may be sufficient to affect the third place in the values for density. This may be due to the effect of lipids in attracting bromobenzene into the cells, to inhomogeneities in the samples of packed cells (cf. Ponder (3)), or to both.

*Difficulties in Determining Hemoglobin*—Before finally adopting the modified Wong method described above, I used the Sahli acid hematin method and Wu's alkali hematin method, both of which turned out to be

unsatisfactory and unreliable as compared with the iron determinations. The errors inherent in these methods are the subject of a separate note (4)

### Results

Table I shows the data obtained for 60 determinations of red blood cell density (gravimetric method) and of hemoglobin concentration (modified Wong iron method). The relation of these two variables to each other, as

TABLE I  
Data from 60 Determinations

Density, mean value	1.0996
s. d.	$\pm 0.0059$
Mean corpuscular Hb concentration, mean value, gm %	33.6*
s. d.	$\pm 1.99$
Correlation coefficient, $r$ , between density and Hb concentration	0.59
s. d.	$\pm 0.085$

\* Corresponding to  $113 \pm 5.7$  mg of Fe

expressed by the correlation between them, constitutes the principal conclusion of this investigation.<sup>3</sup>

<sup>3</sup> An important statistical problem arises in connection with the interpretation of the correlation coefficient, and I am very grateful to Dr Joseph Berkson of the Mayo Clinic for supplying me with the following solution. The problem is this: Suppose that there are two attributes,  $X$  and  $Y$  (e.g., density and hemoglobin concentration), the correlation between which is unity or, more generally,  $r$ . If we measure  $X$  and  $Y$  by any real methods, a series of experimental errors will arise, and these will tend to make it seem that the correlation between  $X$  and  $Y$  is less than it really is. If the standard deviations are known, what will be the effect of the errors on the value of  $r$ ?

Let  $X, Y$  be the variates

$$x = (X - \bar{X}), y = (Y - \bar{Y}) \text{ (deviations from the mean)}$$

$$\sigma_x = \sigma_x, \sigma_y = \sigma_y \text{ (standard deviations of the variates)}$$

$$r^2_{xy} = r^2_{xy} = (\Sigma xy)^2 / \Sigma x^2 \Sigma y^2 = (\Sigma xy)^2 / n^2 \sigma_x^2 \sigma_y^2$$

Introduce an error of measurement  $dx$  into  $X$  and  $dy$  into  $Y$

$$X' = X + dx, Y' = Y + dy \text{ (modified variates)}$$

$$x' = x + dx, y' = y + dy \text{ (deviations from the mean)}$$

If  $dx$  is normally distributed around 0 and uncorrelated with  $dy$  and its s.d. is  $e_x$ , and similarly if for  $y$  the s.d. =  $e_y$ , then

$$r'^2_{xy} = r^2_{x'y'} = \frac{[\Sigma(x + dx)(y + dy)]^2}{n^2 \sigma_x^2 \sigma_y^2} \text{ (the modified correlation)}$$

$$\Sigma(x + dx)(y + dy) = \Sigma xy \text{ (because the } dx, \text{ or } dy \text{ products} = 0)$$

$$\sigma_{x'}^2 = \frac{\Sigma(x + dx)^2}{n} = \frac{\Sigma x^2 + \Sigma dx^2 + 2\Sigma x dx}{n} = \sigma_x^2 + e_x^2$$

$$\sigma_{y'}^2 = \sigma_y^2 + e_y^2$$



## DISCUSSION

The average value for density lies between that of Lindeboom (5) or of Macleod (6), the former giving 1.103 for normal humans, and the latter 1.0989. The most interesting result, however, is the low value for  $r$ , the coefficient of correlation between corpuscular hemoglobin and cell density. This means that the density is determined only in part by the hemoglobin concentration as measured.

One explanation of the result is that since hemoglobin is produced intracellularly from colorless precursors of about the same density as itself<sup>4</sup> a deficiency of the pigment may result in its place being occupied by another protein (the precursor) of about the same density. In this series there occur, for example, two specimens with the same density, 1.099, but with mean corpuscular hemoglobin concentrations of 32.7 and 36.4. The difference, 3.7 per cent, gives an idea of the amount of protein other than hemoglobin which might occupy the place of the pigment in the cells with the lower hemoglobin content. The presence of proteins other than hemoglobin has frequently been suggested on many different grounds and the low correlation between red cell density and mean corpuscular hemoglobin may point in the same direction.

An alternative explanation for the result may be that the weight of hemoglobin indicated by 1 gm. of iron varies, for Morrison (10) found the Fe content in fourteen samples of human hemoglobin to be from 305 to

and so

$$r'_{xy} = \frac{(\sum xy)^2}{n^2(\sigma_x^2 + e_x^2)(\sigma_y^2 + e_y^2)}$$

$$\frac{r}{r'} = \sqrt{\frac{(\sigma_x^2 + e_x^2)(\sigma_y^2 + e_y^2)}{\sigma_x^2 \sigma_y^2}} = \sqrt{\left[1 + \left(\frac{e_x}{\sigma_x}\right)^2\right] \left[1 + \left(\frac{e_y}{\sigma_y}\right)^2\right]}$$

So, as in our specific case (see Table I) if without error the correlation is  $r$  and there is introduced into  $x$  an error for which the standard deviation  $e_x = 0.17\sigma_x$  and into  $y$  an error of measurement for which the s.d. is  $e_y = 0.25\sigma_y$ ,  $r'$  the new correlation will be

$$r' = r \sqrt{\frac{1}{(1 + 0.17^2)(1 + 0.25^2)}} = 0.96r$$

i.e., the correlation coefficient will not be appreciably affected by the errors of measurement associated with the experimental methods.

<sup>4</sup> Using the mean value of all the density determinations and taking the density of a 1 per cent NaCl solution as 1.0075, we get a value for the density of intracellular hemoglobin of 1.32. Svedberg and Pedersen (7) give a value of 0.749 for the partial specific volume of hemoglobin, based on the work of Svedberg and Fåhræus (8). This corresponds to a density of 1.335. Perutz (9) gives a density of 1.242 for the wet crystals.

338 mg. per cent. I have based my calculations on 336 mg. per cent, which does not allow for variation in the Fe content, and it is possible that this factor, plus experimental errors still remaining in the methods, may account for, or at least contribute to, the low correlation found between hemoglobin content and density. For example, the apparent difference between the hemoglobin contents of the two samples with a density of 1.099, supposed to have 32.7 and 36.4 per cent of hemoglobin on the basis of calculation from the iron values, might be due to the iron content of the hemoglobin of the first sample differing from that of the second by about 10 per cent, which, on the basis of Morrison's results, is just possible.

This investigation was carried out under a grant from the Simon Baruch Foundation. I am very grateful to Dr. Donald D. Van Slyke for his many suggestions as to how the work should be carried out and for reading the manuscript.

#### SUMMARY

1. The correlation between red blood cell density and corpuscular hemoglobin concentration, as calculated from the content of iron, is only 0.59, a result which suggests that the place of hemoglobin in the red cell may be taken by a colorless precursor of the same density, that the Fe content of human hemoglobin is inconstant, or both.

2. Difficulty was encountered in applying the Linderström-Lang method of measuring density to packed red blood cells. Successive droplets of packed red cells did not always reach the same equilibrium position, and so seemed to have different densities. The inconstancy was probably largely due to the solubility of cell lipids in the bromobenzene-kerosene medium. A direct gravimetric method proved preferable for cell density.

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# ERRORS AFFECTING THE ACID AND THE ALKALI HEMATIN METHODS OF DETERMINING HEMOGLOBIN

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It was pointed out long ago by Berczeller (1) and by Wu (2) that the color developed in the acid hematin method is affected by non-hemoglobin substances in the plasma, and it was to avoid these that Wu introduced his alkali hematin method, the value of which, however, has never been carefully assessed. Erroneous results with these hematin methods were encountered in the course of another piece of research (3), even when the plasma was removed and the analyses carried out on separated cells, and the errors were not entirely eliminated by the use of Wu's alkali hematin method. The present report presents a study of the nature and magnitude of these errors.

## *Material and Methods*

The material used was either human whole blood or packed human red blood cells, the packing being carried out by spinning for 2 hours at 4000 R.P.M., and the plasma being afterwards removed as completely as possible. Determinations of hemoglobin were made at room temperature by the acid hematin and the alkali hematin methods as described by Peters and Van Slyke (4), the color matching being carried out in a Klett-Summerson photoelectric colorimeter. The results obtained were compared with the result of hemoglobin determinations by a modification of Wong's iron method (Ponder (3)), assuming the iron content of hemoglobin to be 336 mg. per cent.

## *Results*

The principal objections to the acid hematin method when applied to packed cells are (a) that the color takes a long and variable time to develop, and (b) that when fully developed it gives values for the hemoglobin content which do not agree at all well with the results of iron determinations. These defects in the method are illustrated by typical data brought together in Tables I and II.

The first section of Table I shows the long and variable times for the development of color when the method is applied to human packed red cells. The packed cells (20 c.mm.) were added to the acid (10 ml. of 0.1 N HCl), and after mixing, the colorimeter reading was recorded after 15,

30, 60, 90, 120, and 180 minutes. The color is always fully developed at the end of 180 minutes, and so the value observed at that time is denoted by 100 per cent, and is given as gm. of hemoglobin in the last row. It will be apparent that the rate at which color develops is variable, and that, on the average, only about 90 per cent of the full color is developed at the end of 30 minutes, the time at which it is usually supposed to be developed completely.

In Table I are also shown similar results for human whole blood (20 c.mm. added to 5 ml. of 0.1 N HCl). Here the discrepancies are less striking, but the differences in the rate of development of the color are still apparent, particularly in cases in which the hemoglobin content is high.

Table II shows fifteen values for packed cells, arbitrarily selected from

TABLE I

*Varying Rates of Development of Acid Hematin Color by Different Samples of Packed Human Red Blood Cells and of Whole Blood*

The results are expressed in per cent of Hb readings at 180 minutes.

Time	Packed red cells						Whole blood					
min												
15	81 0	89 0	88 3	91.2	87 5	88.0	92 0	96 1	93.2	94 3	96 5	93.1
30	85 5	93 0	92.4	95.0	90 0	92.8	93 6	98 0	94 5	95 2	98 7	95 0
60	91 0	96 0	95 8	97 5	95 5	98 5	96 0	98.7	96 2	96 4	100 0	98 2
90	94 2	99 0	97 0	98 5	97.4	99 5	97 1	99 0	98 0	97 3		100 0
120	98 5	100 0	98 4	99.5	99 5	100 0	98 9	100 0	99.2	99.0		
180	100.0		100 0	100 0	100 0		100.0		100.0	100 0		
Hb, gm. %	36 3	37 7	43.3	37 8	44 4	34.1	14 9	13 8	13 3	12 3	10 8	4 9

a series of 60, in which the hemoglobin content was determined by (a) the modified Wong method, (b) the acid hematin method, and (c) the alkali hematin method. It will be apparent that results obtained by either the acid hematin or the alkali hematin method do not agree well with those by the iron method, nor, indeed, do they agree well with each other.<sup>1</sup> The average discrepancy is 2.4 gm. per cent, or about 6 per cent of the observed (iron) value. This, it should be noticed, is the discrepancy under conditions in which the full color is allowed to develop in both the acid and the alkali hematin determinations; if readings had been made at the end of 30 minutes, as is customary, the discrepancies would have been greater.

<sup>1</sup> Since there is no factor associated with Wu's method, I have used a multiplying constant which makes the mean of all the (60) acid hematin determinations agree with the mean of all the alkali hematin determinations. The colorimetric readings by the alkali hematin method are multiplied by this constant.

The question of the reliability of these hematin methods has already been raised by Peters and Van Slyke (4), and the doubts which they express seem to be wholly justified. It will be noticed that the *large* discrepancies in Table II are due to the acid hematin value being higher than the hemoglobin iron value, which suggests that the color is affected by substances other than hemoglobin.

It has been recognized that plasma contains substances, such as lipids and pigments, which influence the dispersion of hemoglobin derivatives and their color absorption. It is not generally recognized, however, that the

TABLE II

*Comparative Hemoglobin Determinations on Packed Human Red Blood Cells by Iron Method of Wong,\* Acid Hematin Method of Sahli, and Alkali Hematin Method of Wu*

The results express gm. of hemoglobin per 100 gm. of cells.

Iron method	Acid method	Alkali method
41.0	43.0	39.4
39.4	41.8	44.9
36.2	42.6	42.5
32.3	34.0	33.0
36.0	36.4	34.8
28.1	30.1	27.1
40.0	40.2	39.0
33.6	38.6	38.6
29.6	31.2	30.5
37.8	41.2	40.0
31.3	30.6	31.1
36.9	43.4	41.9
38.2	38.1	39.7
33.0	34.4	33.2
32.6	31.4	31.1

\* Wong's method as modified by Ponder (3).

effects remain when the plasma has been removed from the cells, nor that they enter as disturbing factors in the alkali hematin method.

#### SUMMARY

The Sahli acid hematin method and the Wu alkali hematin method give results for hemoglobin content of whole blood or of packed red cells which are often in poor agreement with the results by Wong's iron method. The principal sources of error are the long and variable time required for the full development of color in the hematin methods and the fact that the final color is affected by substances other than hemoglobin, contained both in plasma and cells.

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# A STUDY OF THE NITROGENOUS CONSTITUENTS OF TISSUE PHOSPHATIDES\*

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The problem of the chemical constitution of the phospholipids is of great importance for an understanding of the metabolic function of these substances. The work with radioactive phosphorus has shown that the rates at which newly formed phosphatides were deposited in various organs differed to a remarkable degree and that even the rates of formation of the alcohol-soluble phosphatides ("lecithin") and the alcohol-insoluble phosphatides ("cephalin") of the organism showed differences. In this manner, by comparing the speed of entry of radioactive phosphorus into the phospholipid molecules, it was possible to distinguish between active organs, *e.g.* the liver, and sluggish organs, *e.g.* the brain.

The experiments described here represent an attempt to approach the problem of the composition of tissue phosphatides by a study of the distribution of the principal nitrogenous constituents, especially choline and ethanolamine. By correlating the results concerning the amounts of these bases with the analytical composition of the phospholipids examined it appeared possible to prove or disprove the presence of any hitherto unknown constituents. The analytical tool used was the isotope dilution method (1). The pure bases containing a known amount of the  $N^{15}$  isotope were added to the phosphatide hydrolysis mixture. The isotope dilution found in pure specimens of the compounds isolated from the hydrolysates was a measure of the amounts originally present in the phospholipids. For the isolation of the bases, it appeared advantageous to choose derivatives which could easily be purified and contained no other nitrogen than that of the base. For this reason, choline was isolated as the mercuric chloride double salt, ethanolamine as the 3,5-diiodosalicylate (2). Following the discovery of serine in brain cephalin (3), the amino acid content of the phosphatide preparations used was likewise determined.

In order to obtain representative results, it was sought to avoid, as far as possible, fractionation of the phospholipids by solvent action in the course of the isolation. The preparation of the phosphatides, therefore, was based mainly on the solubility of these substances in petroleum ether

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and their insolubility in acetone. The fractions examined were freed of cerebrosides, sphingomyelin, and water-soluble nitrogenous impurities, and had N:P ratios near 1:1. Specimens obtained from liver, brain, and heart were examined. Some data on phospholipids from lung and from egg yolks are also included.

## EXPERIMENTAL

### *Analytical Procedures*

For analysis all preparations were dried to constant weight *in vacuo* over  $P_2O_5$  at  $60^\circ$  with the exception of the samples used for the determination of the iodine values (4), which were similarly dried at room temperature. Phosphorus was determined gravimetrically according to Pregl-Lieb, total nitrogen by the Kjeldahl procedure. For the estimation of amino nitrogen according to Van Slyke the samples were dissolved in glacial acetic acid, the reaction time with nitrous acid being 5 minutes in all cases. The amino nitrogen values for the heart and liver phosphatides included in a preliminary communication from this laboratory (5) were in error and are corrected by the data presented here. Amino acid nitrogen was titrimetrically determined by the ninhydrin method (6).

The determination of the  $N^{15}$  isotope was carried out in a mass spectrometer (7). The results of the isotope dilution experiments were obtained from the equation  $(a/c - 1)b = x$ , in which  $a$  is the  $N^{15}$  excess in atom per cent and  $b$  the amount of nitrogen in mg. present in the base added to the hydrolysis mixture,  $c$  the  $N^{15}$  excess in atom per cent found in the specimen of the same base isolated from the hydrolysate, and  $x$  the amount of base nitrogen in mg. found by isotope dilution.

### *Preparation of Phosphatides*

*Phosphatides from Liver, Lung, and Brain* (Table I, Preparations 1 to 4)—The isolation and purification of the mixed phosphatides from pig liver, beef lung, and beef brain will be exemplified by the description of one preparation of pig liver phosphatides (Preparation 1, Table I).<sup>1</sup> The ground tissue, weighing 5175 gm., was extracted twice with acetone for 24 hours, 2.5 liters of the solvent being used in each case. The organ powder then was treated with 4.4 liters of petroleum ether (b.p.  $30-60^\circ$ ) for several days and this extraction repeated twice with similar amounts of solvent. The combined petroleum ether extracts were concentrated to a volume of 400 cc. and the solution freed of cerebrosides, etc., by storage

<sup>1</sup> In all cases fresh organs obtained from the slaughter-house were used. All operations were, whenever possible, carried out in a nitrogen atmosphere. The solvents were rectified by distillation.



and centrifugation at 0°. The phosphatides were precipitated from the supernatant by the addition of 4 volumes of chilled acetone. The precipitate was emulsified in 150 cc. of cold physiological saline and 0.5 volume of cold acetone added. The phosphatides, obtained by centrifugation of the chilled mixture, were washed with ice-cold acetone-saline (1:2) and acetone, rapidly dried, and dissolved in 400 cc. of petroleum ether. The solution was cooled, cleared by centrifugation, concentrated to 200 cc., and slowly poured with stirring into 4 volumes of chilled acetone. The flocculent precipitate was allowed to settle in the refrigerator, filtered, washed with chilled petroleum ether-acetone (1:4) and acetone, and dried

TABLE I  
*Tissue Phosphatides*

Preparation No.	Source	Tissue used	Yield of phosphatides	Period of storage	P	Total N	N P	NH <sub>2</sub> -N	NH <sub>2</sub> -N	Amino acid N	Amino acid N	Iodine value
		gm.	gm.	mo.	per cent	per cent		per cent	per cent of total N	per cent	per cent of total N	
1	Pig liver	5175	45.2	0	3.70	1.65	0.99:1	0.87	52.7	0.11	6.7	
				6	3.58	1.57	0.97:1	0.53	33.7			46.2
				10	3.61	1.63	1.00:1	0.46	28.2			36.7
2	" "	2225	26.0	0	3.56	1.65	1.02:1	0.90	54.5			88.8
3	Beef lung	1579	5.9	0	3.80	1.78	1.04:1	1.21	68.0	0.28	15.7	69.7
				7	3.68	1.78	1.07:1	1.03	57.9			47.3
4	" brain	1804	47.2	0	3.66	1.71	1.03:1	1.03	60.2	0.37	21.6	46.6
				7	3.59	1.68	1.03:1	0.85	50.6			43.4
5	Pig heart	3831	29.5	0	3.70	1.58	0.94:1	0.83	52.5	0.11	7.0	86.0
				3	3.63	1.53	0.93:1	0.38	24.8			
				9	3.61	1.57	0.96:1	0.29	19.1			39.7
6	Egg yolk	22 yolks	13.1	0	4.00	1.81	1.00:1	0.76	42.0	0.026	1.4	67.7
				4	3.81	1.78	1.03:1	0.39	21.9			36.8

*in vacuo*. The phosphatides formed a yellowish wax and weighed 45.2 gm. (Preparation 1, Table I).

*Phosphatides from Heart (Table I, Preparation 5)*—Fourteen hearts from freshly slaughtered pigs were cleaned and ground. The minced tissue which weighed 3831 gm. was extracted with acetone and with petroleum ether, as described in the preceding section. The petroleum ether extract was concentrated to a volume of 300 cc., chilled, and freed of insoluble material. The addition of 4 volumes of absolute alcohol to the clear petroleum ether solution produced sedimentation of an oil which on being cooled solidified to a slightly colored soft paste. This material after being dried weighed 104.1 gm., had a low phosphorus content, and was

free of nitrogen (P 0.49, iodine value 58.5). The supernatant from this fraction was concentrated *in vacuo* to a volume of 140 cc. The addition of 2.5 volumes of acetone precipitated 38.7 gm. of an amber-colored wax (P 3.10, N 1.58, amino N 1.01, iodine value 86.6). This phosphatide mixture was emulsified in 250 cc. of physiological saline and precipitated with 125 cc. of acetone. The petroleum ether solution of the resulting precipitate was cleared by centrifugation in the cold and the phosphatides were precipitated with acetone. They formed a light brown waxy substance, weighing 29.5 gm. (Preparation 5, Table I).

*Phosphatides from Egg Yolk* (Table I, Preparation 6)—The yolks from twenty-two eggs, freed of membranes and chalazae, were mixed with 200 cc. of physiological saline and slowly poured with stirring into 2400 cc. of a hot mixture of equal parts of alcohol and ether. After the mixture had stood overnight at room temperature, the coagulated proteins were filtered off, and the filtrate was concentrated to 1 liter and poured into 3 volumes of chilled acetone. The solution of the resulting semisolid precipitate in 300 cc. of petroleum ether was freed of moisture by means of anhydrous sodium sulfate and of cerebrosides by freezing. The phosphatides, obtained by acetone precipitation, were further purified by flocculation from their emulsion in physiological saline, etc., as described in the preceding sections. The final product weighed 13.1 gm. and formed a light tan-colored powder (Preparation 6, Table I).

#### *Effect of Storage on Phosphatide Composition*

A number of phosphatide preparations were stored in the refrigerator in well stoppered bottles in a nitrogen atmosphere and after considerable periods again subjected to analysis with the results shown in Table I. During that time the substances changed in appearance: the yellow soft waxes became dark brown and brittle.

#### *Determination of Ethanolamine and Choline by Isotope Dilution Method*

The phosphatide preparations discussed below were all freshly prepared. The isotopic bases used, *viz.* the hydrochlorides of ethanolamine and choline, both contained 2.00 atom per cent  $N^{15}$  excess.

*Pig Liver Phosphatides*—For hydrolysis 5.039 gm. of Preparation 1 (Table I) were refluxed with 50 cc. of 1 N hydrochloric acid for 18 hours. At the end of the hydrolysis 46 cc. of an aqueous ethanolamine hydrochloride solution containing 17.7 mg. of labeled nitrogen were added to the hydrolysis mixture which then was again heated to boiling for a short time. The fatty acids were removed by filtration from the chilled mixture and boiled with 1 N hydrochloric acid. The combined aqueous filtrates were evaporated to complete dryness *in vacuo*. The residue was dissolved in water and the solution neutralized by means of solid silver carbonate.

After addition of 2 gm. of basic lead acetate, the precipitate was removed, the filtrate freed of heavy metals by means of hydrogen sulfide, acidified with hydrochloric acid, and evaporated to complete dryness *in vacuo*. The residue was dissolved in absolute alcohol to give a volume of 100 cc. (This solution was found to contain a total of 81.6 mg. of total nitrogen and 40.4 mg. of amino nitrogen. After correction for the ethanolamine added, the recovery corresponded to 76.9 per cent of total nitrogen, but to only 51.8 per cent of amino nitrogen originally present in the sample used for hydrolysis.) The alcoholic solution of the base chlorides (98 cc.) was evaporated to dryness *in vacuo*, the residue dissolved in 2.5 cc. of water, and this solution mixed with calcium oxide. The isolation of ethanolamine as diiodosalicylate was carried out, as described in a recent publication from this laboratory ((2) p. 496). The crude *ethanolamine 3,5-diiodosalicylate* weighed 891.3 mg. (corresponding to 69.8 per cent of the amino nitrogen present in the alcoholic solution of the base chlorides). Two crystallizations from ligroin-absolute alcohol (4:1) yielded 466.9 mg. of white needles melting (with decomposition) at 196° (corrected).

$C_9H_{11}NO_4I_2$  (451.0). Calculated. I 56.3, N 3.1

Found. " 56.5, " 3.0, N<sup>15</sup> excess 0.882 atom %

The phosphatide hydrolysis mixture was by the isotope dilution method found to contain 22.45 mg. of ethanolamine nitrogen. The mixed liver phosphatides examined, therefore, contained 0.445 per cent of ethanolamine nitrogen.

In another experiment 4.952 gm. of Preparation 1 (Table I) were refluxed with 100 cc. of 6 N hydrochloric acid for 45 hours. The filtrate from the fatty acids was adjusted to a volume of 200 cc. (This solution contained a total of 71.2 mg. of total nitrogen and 31.2 mg. of amino nitrogen, corresponding to recoveries of 87.1 and 72.4 per cent of the original amounts of total and amino nitrogen respectively.) To 185.5 cc. of this solution labeled choline hydrochloride (corresponding to 2.65 mg. of labeled nitrogen) was added. The solution was then treated as described in the preceding paragraph. The base hydrochlorides were adsorbed by calcium oxide; the mixture was first extracted with ether, in order to remove the ethanolamine, followed by extraction with absolute alcohol. From the alcoholic extract *choline* was isolated as the *mercuric chloride double salt* ((2) p. 497). The product weighed 1.419 gm. and after recrystallization from water melted (with decomposition) at 253–255° (corrected).

$C_5H_{14}NOCl \cdot 6HgCl_2$  (1768.8). Calculated. N 0.79

Found. " 0.78, N<sup>15</sup> excess 0.129 atom %

The amount of non-amino nitrogen present in the hydrolysis mixture to which the isotopic choline was added was 37.1 mg. By the isotope dilu-

tion method 38.4 mg. of choline nitrogen were found. It, therefore, may be concluded that the solution of the bases obtained by hydrolysis of the liver phosphatide sample contained all its non-amino nitrogen in the form of choline.

**Beef Brain Phosphatides**—The investigation of this phosphatide preparation was carried out by a somewhat different procedure. To a suspension of 5.0409 gm. of Preparation 4 (Table I) in 100 cc. of 2 N  $\text{H}_2\text{SO}_4$  ethanolamine hydrochloride (containing a total of 8.03 mg. of labeled nitrogen) and choline hydrochloride (containing a total of 1.99 mg. of labeled nitrogen) were added. The mixture was heated under a reflux for 40 hours, the fatty acids were removed and washed, and the combined filtrate and washings diluted to a volume of 200 cc. (This solution was found to contain a total of 86.2 mg. of total nitrogen and 58.8 mg. of amino nitrogen, corresponding, after correction for the added isotopic bases, to recoveries of 89.6 and 98.1 per cent respectively.) 194 cc. of the solution were made alkaline by the addition of finely powdered  $\text{Ba}(\text{OH})_2$ , neutralized by means of  $\text{CO}_2$ , and, after addition of 2 volumes of alcohol, freed from the precipitate. The filtrate was acidified with dilute  $\text{HCl}$ , evaporated to complete dryness *in vacuo*, and the residue dissolved in 100 cc. of absolute alcohol. (The alcoholic solution of the base chlorides contained a total of 68.7 mg. of total nitrogen.) The derivatives of ethanolamine and choline were isolated, as described in the preceding section. The recrystallized ethanolamine 3,5-diiodosalicylate melted (with decomposition) at 197–199° (corrected).

$\text{C}_8\text{H}_{11}\text{NO}_4\text{I}_2$  (451.0). Calculated. N 3.1

Found. " 3.0,  $\text{N}^{15}$  excess 0.348 atom %

The recrystallized choline-6 $\text{HgCl}_2$  double salt melted (with decomposition) at 251–253° (corrected).

$\text{C}_8\text{H}_{11}\text{NOCl} \cdot 6\text{HgCl}_2$  (1768.8). Calculated. N 0.79

Found. " 0.80,  $\text{N}^{15}$  excess 0.209 atom %

On the basis of the isotope analyses the brain phosphatide preparation contained 0.756 per cent of ethanolamine nitrogen and 0.398 per cent of choline nitrogen.

**Pig Heart Phosphatides**—To a suspension of 5.0317 gm. of the phosphatide sample (Preparation 5, Table I) in 120 cc. of 2 N  $\text{H}_2\text{SO}_4$  the isotope-containing bases (13.0 mg. of labeled ethanolamine nitrogen, 2.72 mg. of labeled choline nitrogen) were added and the mixture was hydrolyzed for 48 hours. The filtrate from the fatty acids was adjusted to pH 10 by the addition of finely powdered  $\text{Ba}(\text{OH})_2$ . After 1 hour the mixture was neutralized by means of  $\text{CO}_2$  and freed of the precipitate. The addition of 2 volumes of absolute alcohol to the filtrate precipitated 313.3 mg. of a barium salt which was almost free of nitrogen (P 4.6, N 0.18). The

filtrate from this precipitate was acidified with dilute HCl, completely evaporated *in vacuo*, and the residue was dissolved in 100 cc. of absolute alcohol. (The alcoholic solution contained a total of 61.3 mg. of total nitrogen, corresponding to a recovery of 64.4 per cent of the amount originally present.) From 98 cc. of the alcoholic solution the bases were isolated in the usual manner. *Ethanolamine 3,5-diiodosalicylate* weighed, after recrystallization, 533.3 mg. and melted (with decomposition) at 197–198° (corrected).

$C_9H_{11}NO_4I_2$  (451.0). Calculated. I 56.3, N 3.1  
Found. " 56.3, " 3.1,  $N^{15}$  excess 0.598 atom %

The recrystallized *choline-mercuric chloride double salt* weighed 460 mg. and melted (with decomposition) at 250–251° (corrected).

$C_9H_{11}NOCl \cdot 6HgCl_2$  (1768.8). Calculated. N 0.79  
Found. " 0.84,  $N^{15}$  excess 0.257 atom %

The isotope analyses revealed the presence in the heart phosphatide preparation of 0.605 per cent of ethanolamine nitrogen and of 0.367 per cent of choline nitrogen.

In another experiment, designed to determine the nature of the amino nitrogen fraction in the final alcoholic solution of base hydrochlorides derived from the hydrolysis of the phosphatide, 11.66 gm. of the heart phosphatide specimen (Preparation 5, Table I) were hydrolyzed with 250 cc. of 2 N  $H_2SO_4$ . The hydrolysis mixture was treated as described above and the base hydrochlorides were dissolved in 100 cc. of absolute alcohol. This solution contained a total of 106.0 mg. of total nitrogen and 42.8 mg. of amino nitrogen, corresponding to recoveries of 57.5 and 44.2 per cent respectively. To 35 cc. of this alcoholic solution (containing a total of 14.98 mg. of amino nitrogen) 20 cc. of an aqueous solution of isotopic ethanolamine hydrochloride (containing a total of 2.81 mg. of labeled nitrogen) were added and ethanolamine isolated in the customary way. The recrystallized *ethanolamine 3,5-diiodosalicylate* weighed 94.5 mg. and melted (with decomposition) at 197° (corrected).

$C_9H_{11}NO_4I_2$  (451.0). Calculated. I 56.3, N 3.1  
Found. " 56.4, " 3.0,  $N^{15}$  excess 0.327 atom %

The sample subjected to the analysis by isotope dilution was found to contain 14.37 mg. of ethanolamine nitrogen, corresponding to 96.0 per cent of the total amino nitrogen present.

#### DISCUSSION

The results of the isotope dilution experiments carried out with phosphatides from liver, brain, and heart are summarized in Table II which extends and corrects some of the data contained in a preliminary note (5).

It will be seen that only about two-thirds of the amino nitrogen present in the liver phosphatides could be accounted for as ethanolamine or amino acid; the non-amino nitrogen of this preparation, on the other hand, appears to exist entirely in the form of choline. All amino nitrogen present in brain phosphatides and practically all amino nitrogen occurring in the heart phosphatides could be identified as ethanolamine and amino acid. The specimens from brain and heart contained, however, a large portion of unidentified non-amino nitrogen. In the brain phosphatides 86 per cent of the total nitrogen present, and in the heart phosphatides 69 per cent were identified. Discussion of the nature of the unknown nitrogenous constituents would at present be largely speculative, but it can be pointed

TABLE II  
*Nitrogen Distribution in Phosphatides*

Preparation No.*	Source	Amino N in phosphatide	Ethanolamine N by isotope dilution		Amino acid N		Unidentified amino N	Non-amino N in phosphatide	Choline N by isotope dilution		Unidentified non-amino N
			per cent of phosphatide	per cent of amino N	per cent of phosphatide	per cent of amino N			per cent of phosphatide	per cent of non-amino N	
1	Pig liver	0.87	0.45	51.7	0.11	12.7	35.6	0.78	†	†	†
4	Beef brain	1.03	0.76	73.8	0.37	35.9	0	0.68	0.34	50.0	50.0
5	Pig heart	0.83	0.61†	73.5†	0.11	13.3	13.2	0.75	0.37	49.3	50.7

\* The numbers refer to Table I.

† All non-amino N contained in the hydrolysate was found by the isotope dilution method to be choline N.

‡ All amino N contained in the alcoholic solution of the base hydrochlorides derived from the hydrolysate was found by the isotope dilution method to be ethanolamine N.

out that the presence of unidentified non-amino compounds may in part be only apparent. The presence of acid amide linkages between the free amino group of ethanolamine (or a hydroxyamino acid) and either an organic acid or the free acidic hydroxyl of the phosphoric acid contained in the phosphatides would serve to simulate the presence in the intact compound of non-amino nitrogen which on hydrolysis would give rise to amino nitrogen. While there are indications of the occurrence of such linkages, the evidence is not sufficient, and, in any event, this assumption would merely shift the uncertainty from the group of nitrogenous substances containing non-amino nitrogen to that of compounds containing free amino groups.

The amino acid content of phosphatides, summarized in Table I, deserves some comment. The phosphatides from beef brain contained con-

siderable amounts of amino acid, a finding which confirms the results of Folch and Schneider (3). The contribution of the amino acid nitrogen to the total amino nitrogen was smaller in the phosphatide sample examined here (Preparation 4, Table I) than in purified brain cephalin (3), since in the present experiment fractionation (*i.e.* the removal of the alcohol-soluble ethanolamine phosphatide), which invariably takes place during the preparation of cephalin free of lecithin, was avoided. Considerable amounts of amino acid phosphatides were found in lung, whereas the phospholipids from liver and heart contained much smaller quantities. The egg yolk phosphatides, peculiarly enough, were free of amino acid. The obvious complexity of the composition of tissue phosphatides will make it important to reinvestigate such questions as the relative speed of synthesis of the individual organ phosphatides and the action of snake venoms in the light of recent findings.

The experiments on the effect of storage on the analytical composition of phosphatides, likewise summarized in Table I, revealed profound changes. The iodine values, as was to be expected, and, more remarkably, the amino nitrogen values showed a considerable drop (with the exception of the specimen from brain), whereas the figures for phosphorus and total nitrogen remained unchanged. This seeming decrease in amino nitrogen obviously was not due to the destruction during storage of the constituents containing free amino groups, but to a masking of these groups, perhaps as the result of amide formation, as discussed above, since by the hydrolysis of aged specimens much more amino nitrogen was produced than corresponded to the intact phosphatide. A liver phosphatide sample (stored for 6 months), for instance, after hydrolysis yielded 19 per cent more amino nitrogen than was found in the unhydrolyzed preparation, and a heart phosphatide specimen (stored for 3 months) similarly produced an excess of 33 per cent of amino nitrogen.

The authors wish to thank Mr. A. Bendich for experimental assistance and Mr. W. Saschek for a number of halogen analyses.

#### SUMMARY

1. The distribution of ethanolamine and choline in purified phosphatides from liver, brain, and heart was determined by the method of isotope dilution with  $N^{15}$ . The amino acid content of these phosphatides as well as of samples from lung and egg yolk was likewise determined.

2. In a preparation of pig liver phosphatides 35.6 per cent of the amino nitrogen could not be characterized either as ethanolamine or amino acid. All of the non-amino nitrogen in a hydrolysate of this phosphatide was found to be present as choline.

3. In a preparation of beef brain phosphatides all the amino nitrogen

could be identified as ethanolamine and amino acid, whereas only 50 per cent of the non-amino nitrogen was accounted for as choline.

4. In a preparation of pig heart phosphatides 86.8 per cent of the amino nitrogen was accounted for as ethanolamine and amino acid; only 49.3 per cent of the non-amino nitrogen could be characterized as choline.

5. The phosphatides from brain and lung were found to have the highest amino acid content. The egg yolk phosphatides were free of amino acid.

6. Data on the effect of storage on phospholipid composition and a discussion of some of the implications of the experimental results are included.

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# THE ISOLATION AND CRYSTALLIZATION OF PLANT VIRUSES AND OTHER PROTEIN MACRO MOLECULES BY MEANS OF HYDROPHILIC COLLOIDS

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## PLATE I

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Tobacco mosaic virus is a nucleoprotein of high molecular weight (1). The question of the mode of linkage between the phosphorylated prosthetic group, nucleic acid, and the protein component of the virus is a problem common to many enzymes whose prosthetic groups are also phosphorylated. The lability of the linkage between the phosphatide prosthetic group and the protein, in the case of the thromboplastic protein from lungs (2), has been demonstrated by the action of heparin in displacing the phosphatide and in altering the physiological activity of this particular enzyme by attaching itself to the protein carrier (3). It was considered to be of interest to determine whether heparin might similarly replace the nucleic acid in tobacco mosaic virus.

It was observed, however, that the addition of heparin to virus solutions resulted in the production of needle-shaped paracrystals, without the displacement of nucleic acid from the protein moiety. Additional experiments have demonstrated a new type of reaction between very large protein molecules, of which tobacco mosaic virus is an example, and hydrophilic colloids, such as heparin (4). Many proteins of animal and plant origin having molecular weights greater than  $10^6$  were precipitated by these hydrophilic substances. Furthermore, some of the precipitated proteins, depending on their particular properties, state of purity, and the specific precipitant, assumed crystalline form. The studies were extended to demonstrate certain practical possibilities, such as the isolation of tobacco mosaic virus from infectious juice and the separation of two viruses. In addition, experiments were undertaken to attempt to clarify the mechanism of precipitation.

## EXPERIMENTAL

*Precipitation and Crystallization of Plant Viruses*—Virus preparations were purified by means of differential centrifugation. We are indebted to

\* Fellow in the Medical Sciences of the National Research Council.

Dr. C. A. Knight for preparations of strains of tobacco mosaic virus (TMV), including the green aucuba, yellow aucuba, masked, J14D1, and Holmes rib-grass strains, and cucumber mosaic virus 4 (5). Tobacco mosaic, tomato bushy stunt (6), and tobacco necrosis viruses were also studied. Virus solutions were used at a concentration of 4 mg. per cc. in 0.1 M phosphate buffer (pH 7.1). The precipitant, heparin, hyaluronic acid, or chondroitinsulfuric acid, was also dissolved in this buffer in a wide range of concentrations. The addition of an equal volume of the colloidal solution to the virus solution and the appearance of the precipitated virus after the mixture had stood 15 minutes at room temperature permitted the grouping of the viruses according to their precipitability with these reagents. The results are summarized in Table I.

TABLE I  
*Precipitability of Plant Viruses by Means of Heparin, Hyaluronic Acid,  
and Chondroitinsulfuric Acid*

Concentration of colloidal anion	Virus
<i>per cent</i>	
2.5	Tomato bushy stunt Tobacco necrosis
1	Cucumber mosaic 4 Holmes rib-grass
>0.5	Masked J14D1
<0.5	Green aucuba Tobacco mosaic Yellow aucuba

The precipitation of some preparations, particularly of TMV and Holmes rib-grass virus, was inhibited by the presence of electrolyte. While the groups represented by TMV and tomato bushy stunt virus were always separated by a difference of precipitability, some preparations of viruses in the intermediate groups have exhibited precipitability which would place them in other groups. The effect of electrolytes will be considered later in greater detail.

The individual molecules of TMV, its strains, and cucumber mosaic virus 4, among others, are highly asymmetric (7). After solutions of these viruses were adjusted to the appropriate concentration of heparin, a crystalline sheen, readily dispersed by stirring, appeared in the virus solutions within 1 minute. Microscopic examination of precipitated virus preparations of this type after the addition of heparin revealed the formation of paracrystals similar to those produced by the addition of salts or acids (8, 9). Crystallization of these viruses by means of excess reagent pro-

duced extremely elongated particles. Figs. 1 to 6 demonstrate the two-dimensional orientation produced by the addition of small amounts of heparin to the asymmetric viruses.

It appeared desirable to determine the composition and the biological activity of one of the crystalline precipitates which were readily soluble in water or buffer. Since a method of purification of a precipitate involving re-resolution might dissociate a loosely bound compound, a precipitate of TMV was analyzed directly, despite its content of small amounts of mother liquor. To 7 cc. of a salt-free solution of TMV at a concentration of 19.2 mg. per cc. were added 7 cc. of a solution containing 9.8 mg. of heparin per cc. The pH of the mixture was approximately 7. After 15 minutes at room temperature, the precipitate was centrifuged for 30 minutes at 2800 R.P.M. and the supernatant liquid was removed. The dried precipitate weighed 135 mg. This material and the original TMV prepara-

TABLE II

*Comparison of Tobacco Mosaic Virus (TMV) and Heparin-Precipitated Virus*

Analyses	TMV	Heparin-TMV ppt.
S, % .	0.45	0.48
Ash, %	2.06	2.16
N, %	15.35	15.30
P, % . .	0.44	0.40
$S_{20}^{*}$ ..	180; 200	187; 201
Virus activity at $10^{-5}$ gm. per cc.†	12	13

\* In Svedberg units, i.e.  $10^{-13}$  cm. per second per unit field.

† Average number of lesions per half leaf on plants of *Phaseolus vulgaris* L.

tions were analyzed by Dr. A. Elek of the Rockefeller Institute. A precipitate, similarly formed, was redissolved in 0.1 M phosphate buffer at pH 7.1 and was examined at a concentration of 3.8 mg. per cc. in the analytical ultracentrifuge by Dr. M. A. Lauffer. The preparation showed the same double boundary and sedimentation constants as the original virus solution. Tobacco mosaic virus in a heparin solution of concentration insufficient to precipitate the virus completely was examined in the electron microscope through the courtesy of Dr. T. F. Anderson, RCA Fellow of the National Research Council. The observed virus particles appeared similar to those of normal TMV (10).

Another preparation of TMV precipitated by means of heparin was tested for virus activity at  $10^{-5}$  gm. per cc. on twenty-four half leaves of *Phaseolus vulgaris* L. (11) against a standard of the original TMV solution. The redissolved virus protein showed no apparent loss of activity. The analytical data are summarized in Table II. The heparin preparation had

a sulfur and nitrogen content of 10.77 per cent and 2.08 per cent, respectively. From the S and N determinations, the maximal content of heparin in the precipitate might be 0.3 per cent. The analytical techniques so far developed are inadequate to indicate whether the small amounts of heparin are chemically combined or are a physically admixed impurity.

The considerable difference of concentrations of heparin required to precipitate tobacco mosaic virus and tobacco necrosis virus was employed in the successful separation of these viruses from the juice of tobacco plants bearing a mixed infection (12). The procedure used will be presented in a later paper.

It was of interest to attempt the crystallization of tomato bushy stunt virus, which crystallizes from solutions of ammonium sulfate as dodecahedra (13, 6). 4 cc. of an 8 per cent heparin solution were added to 3 cc. of a solution of tomato bushy stunt virus containing 7.1 mg. per cc. After 24 hours at 22°, a small amount of precipitate appeared, of which one-third consisted of non-birefringent prisms. On being chilled at 4° for another 24 hours, the amorphous material redissolved, while the crystals already present acted as seed for the further crystallization of the virus. The crystal form of this virus is shown in Fig. 7.

The precipitate was centrifuged at 4° and retained the same type of crystallinity after being washed with cold 26 per cent ammonium sulfate solution. The residue readily dissolved in 3 cc. of water and the solution was centrifuged. The supernatant liquid was analyzed for carbohydrate, phosphorus, and biological activity. The activity of this solution, estimated at  $1.4 \times 10^{-4}$  gm. per cc. by the half leaf method on *Nicotiana glutinosa* L., was not significantly different from that of the original preparation at the same concentration. Carbohydrate was determined by the orcinol reaction (14), a method in which heparin yields 50 per cent of its weight as carbohydrate and virus nucleic acid 66 per cent of its carbohydrate content (15). The solution contained 0.090 mg. of carbohydrate and 0.027 mg. of phosphorus per cc. (16). If heparin were combined with virus, the compound should have a high carbohydrate content. The carbohydrate to phosphorus ratio of tomato bushy stunt virus is normally 1, due to its content of nucleic acid (6). Since a carbohydrate to phosphorus ratio of 0.85 was obtained by correcting the apparent pentose content by the factor 1.5, it was concluded that very little or no heparin was bound in the prisms described above.

*Isolation and Recovery of Crystalline Tobacco Mosaic Virus from Tobacco Juice*—The juice of a mosaic-diseased Turkish tobacco plant was centrifuged at 3000 R.P.M. for 30 minutes and the clarified juice was subjected to four precipitation cycles at concentrations of heparin of 2, 1.5, 1, and 0.5 per cent, respectively. After the third cycle, the virus solution was completely colorless and could be crystallized readily.

19.0 mg. of TMV were added to 10 cc. of juice from a healthy Turkish tobacco plant. Four precipitation cycles as described above resulted in the recovery of 75 per cent of the added virus in the usual crystalline form.

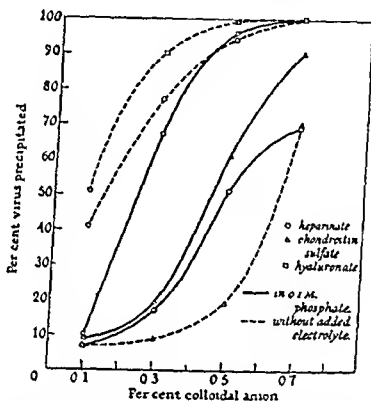
*Other Colloids Effective in Crystallization of Tobacco Mosaic Virus*—The following compounds in order of decreasing effectiveness crystallized purified TMV in 0.1 M phosphate buffer at pH 7.1: agar, gelatin, gum ghatti, gum arabic, thymus nucleic acid, soluble starch. The approximate minimal effective concentrations were 0.25, 0.5, 1.0, 1.0, 1.0, and 3 per cent, respectively. Since the isolation of the virus from juice by means of heparin appears to be specific and results in good yields, duplication of the isolation with one of these inexpensive reagents would be of considerable value. Although none of the practicable compounds was as effective as heparin or hyaluronic acid, gum arabic, for one, was of value in concentrating virus rapidly. Starting with infectious juice, four precipitation cycles at concentrations of gum arabic of 4, 3, 2, and 1 per cent, respectively, resulted in colorless amorphous virus preparations containing excess carbohydrate. The carbohydrate was readily removed by one sedimentation of the virus in the ultracentrifuge to yield a crystallizable preparation.

*Importance of Charge of Colloid and Added Electrolyte in Precipitating TMV*—Heparin (4), hyaluronic acid (17), and chondroitinsulfuric acid (18) are similar in structure. Their basic unit, consisting of acetylated hexosamine linked to hexuronic acid, is polymerized to an unknown extent. In the case of heparin, the unit is a polysulfuric acid ester, while chondroitinsulfuric acid contains a mole of sulfuric acid per mole of hexosamine. Hyaluronic acid does not contain sulfuric acid. Although all three compounds are anionic at pH 7, their magnitude of charge decreases in accord with their content of sulfuric acid in the order heparinate > chondroitinsulfate > hyaluronate. Inasmuch as the salting-out of proteins by such compounds as ammonium sulfate is a function of the ionic strength of the salt solution (19), and hence of the magnitude of charge, a quantitative study of the precipitation of TMV by these colloidal anions should indicate the relationship of the mechanism of precipitation to the common salting-out procedure.

0.2 cc. aliquots of TMV containing 0.67 mg. of N were added to 0.8 cc. of solutions of the three colloidal anions at concentrations varying from 0.1 to 0.7 per cent in water at about pH 7 or 0.1 M phosphate buffer at pH 7.1. The mixtures were kept at 4° for 16 hours and then centrifuged. The precipitates were analyzed for nitrogen by the Kjeldahl method. From the data, which are summarized in Text-fig. 1, two conclusions may be drawn: (1) the precipitation of TMV by the three colloids is apparently not a function of the magnitude of charge of the particular colloid, and hence the mechanism of the reaction appears to be dissimilar to salting-out, and (2) whereas precipitation of this preparation of TMV by heparinate

and hyaluronate is inhibited by 0.1 M phosphate, electrolyte assists the precipitation by means of chondroitinsulfate. The same effects were observed with 0.1 M sodium chloride. It is not considered that the slight differences of pH caused the gross differences in precipitability, since precipitation of TMV by 0.5 per cent heparinate was approximately maximal over a wide pH range.

The precipitation of TMV by starch did not occur in the absence of electrolyte. Maximal precipitation occurred with 0.1 M phosphate and 5 per cent starch, or with 0.5 M phosphate and 2 per cent starch. The precipitation of TMV by gelatin was greatly aided by added electrolyte, and the inhibition by 0.1 M electrolyte in the case of heparinate, hy-



TEXT-FIG. 1. Precipitation of tobacco mosaic virus at pH 7 by means of colloidal anions varying in magnitude of charge in the order heparinate > chondroitinsulfate > hyaluronate.

aluronate, and gum arabic was overcome by the addition of more electrolyte.

*Action of Hyaluronidase on TMV Precipitated by Hyaluronic Acid*—The products formed by the hydrolysis and depolymerization of hyaluronic acid by hyaluronidase (20) failed to precipitate TMV. When 0.5 cc. of a solution of TMV containing 5.02 mg. per cc. was mixed with 0.5 cc. of 1 per cent hyaluronic acid, and the system made 0.1 M with respect to dipotassium phosphate to give a pH of 6.0, an immediate precipitate of virus formed. Incubation of this mixture for 18 hours at 37° did not result in solution of the virus. When, however, 1 mg. of hyaluronidase flavianate was added to a similar tube and the mixture incubated for 18 hours at 37°, a clear solution resulted. The virus could be reprecipitated from this solution by the addition of hyaluronic acid.

*Precipitability of Animal Proteins and Crystallization of a Snail Hemocyanin by Means of Heparin*—It was observed that certain proteins of comparatively low molecular weight, such as edestin, gelatin, and pepsin, were not precipitated by heparin at concentrations up to 10 per cent. Very large proteins of animal origin, such as the cytoplasmic inclusions, animal viruses, and hemocyanins, were then tested. None of the materials was as readily precipitated as TMV. In Table III, which summarizes the results, the proteins are arranged in order of increasing molecular weight, the range being approximately  $10^6$  to  $10^8$ . A close relation between the molecular weight of the proteins studied and their precipitability was not indicated.

TABLE III  
*Precipitation of Animal Protein Macro Molecules by Heparin*

Preparation	Precipitability*		
	Immediate	1 hr.	24 hrs
Influenza virus	10, —	10, —	10, —
<i>Limulus</i> hemocyanin	5, —	5, —	5, —
Snail hemocyanin ( <i>Viviparus malleatus</i> )	5, —	5, +	4, +
<i>Buseycon</i> hemocyanin	5, +	3 7, +	3, +
Guinea pig liver particles (pink)	6 6, +	6, +	5, +
<i>Amphiuma</i> liver particles (small)	6 6, +	5, +	
Beef lung particles	10, +	5, —	5, +
Guinea pig liver particles (white)	10, —	10, —	10, —
Mouse kidney particles	10, —	10, —	10, +

\* The numerical figure represents the percentage of heparin concentration which was tested + represents precipitation, — represents absence of precipitation.

Of the nine preparations examined in this manner, only the hemocyanin of a snail, *Viviparus malleatus*, was observed to crystallize. To 0.1 cc. of protein solution containing 0.50 mg. of N per cc. was added 0.1 cc. of a 10 per cent heparin solution. When this solution was allowed to stand overnight at room temperature, small hexagonal plates shown in Fig. 8 separated. This material was soluble in water and in dilute heparin solutions. The crystallization was repeated readily.

#### DISCUSSION

The evidence which has been presented does not permit a well founded hypothesis to be advanced concerning the reason for the precipitation. In the case of the asymmetric tobacco mosaic virus and the spherical tomato bushy stunt virus, there is no evidence that heparin combines with either protein. In addition, measurements of the surface tensions and viscosities

of solutions from which the virus has been crystallized have not indicated a correlation between precipitability and these physical properties.

Since the pH at which the precipitation may be carried out is one at which both protein and colloid are anionic, this phenomenon appears to be different from a salt-like combination of colloids which usually takes place when the net charges of the two substances are opposite in sign. Examples of this latter type of reaction are the chondroitinsulfate-egg albumin system (21) and the TMV-ribonuclease precipitation (22). There is electrophoretic evidence, however, of interaction between heparin and serum albumin without precipitation at a pH at which both substances are anionic (23). The comparative unimportance of the magnitude of the charge of the colloid has been demonstrated by several different types of experiments: (1) the greater efficacy of hyaluronate over chondroitinsulfate, (2) the inability of the depolymerization products of hyaluronate to precipitate TMV, and (3) the dual requirement of a comparatively uncharged carbohydrate such as soluble starch and electrolyte to crystallize TMV. However, it is clear that the ion atmosphere of the medium cannot be neglected.

It is possible that the precipitation and crystallization *in vitro* of asymmetric macro molecules and other proteins may be similar to many types of phenomena observed in normal and pathological conditions. It is known that hyaluronic acid exists in relatively high concentrations in fluids associated with various tumors, some of which have a virus as causative agent (24, 25), and that considerable amounts of heparin are apparently localized in the cells of the reticulo-endothelial system (26, 27) and are liberated into the blood stream in peptone shock and anaphylactic shock (4). These facts would lead one to expect that these substances may play a rôle in the orientation of cytoplasmic particles and macro molecular solutes within the cell, in the sedimentation rate<sup>1</sup> and clumping of blood cells and bacteria, and in connection with other problems in which the organization of living tissues in definite spatial relationships is of importance.

The author is greatly indebted to Dr. W. M. Stanley for many suggestions during the course of this work. He likewise acknowledges indebtedness to Roche-Organon, Inc., for an ample supply of heparin, to Dr. K. Meyer of Columbia University for preparations of hyaluronic acid and hyaluronidase flavianate, to Dr. L. A. Chambers and Dr. W. Henle of the

<sup>1</sup> Dr. T. Shedlovsky has recently informed the author that the sedimentation rate of red blood cells is markedly increased by pneumococcus polysaccharides and hyaluronic acid. The effect produced by hyaluronic acid may be reversed by hyaluronidase.



University of Pennsylvania for preparations of influenza virus and mouse kidney particles, to Dr. W. C. Boyd of Boston University for the hemocyanin preparations, to Dr. A. Claude of the Rockefeller Institute for preparations of guinea pig liver and *Amphiuma* liver particles, and to Dr. E. Chargaff of Columbia University for a preparation of beef lung particles.

#### SUMMARY

The precipitation of various protein macro molecules, such as plant viruses, hemocyanins, etc., by means of hydrophilic colloids is described. The applications of this reaction, in (1) the crystallization of tobacco mosaic virus (TMV) and its strains, tomato bushy stunt virus, and a snail hemocyanin, (2) the separation of two plant viruses, and (3) the rapid isolation of crystalline TMV from infectious juice, are described. Tomato bushy stunt virus was found to assume a new crystalline form in the presence of heparin. The precipitation of TMV and tomato bushy stunt virus by means of heparin at pH 7 does not seem to involve chemical combination. The effect of electrolytes and the charge of three colloids of similar structure on the precipitation of TMV was examined. Other properties of the colloids are considered in relation to the precipitation of TMV.

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## EXPLANATION OF PLATE I

- FIG. 1. Crystallization of tobacco mosaic virus in 0.50 per cent heparin solution.  $\times 716$ .
- FIG. 2. Crystallization of yellow aucuba virus in 0.50 per cent heparin solution.  $\times 716$ .
- FIG. 3. Crystallization of green aucuba virus in 0.67 per cent heparin solution.  $\times 716$ .
- FIG. 4. Crystallization of J14D1 virus in 0.67 per cent heparin solution.  $\times 716$ .
- FIG. 5. Crystallization of masked virus in 0.67 per cent heparin solution.  $\times 716$ .
- FIG. 6. Crystallization of cucumber mosaic virus 4 in 2.50 per cent heparin solution.  $\times 716$ .
- FIG. 7. Crystallization of tomato bushy stunt virus in 4.6 per cent heparin solution.  $\times 716$ .
- FIG. 8. Crystallization of snail hemocyanin in 5.0 per cent heparin solution.  $\times 716$ . (Photographs by J. A. Carlile.)

of the usual methods which employ heat. The advantages of this procedure for the estimation of sodium in biological materials were pointed out by Sobel, Kraus, and Kramer (6). In the case of potassium, wet ashing at a relatively low temperature cannot be employed because ammonium salts must be removed. In dry ashing losses occur due to the relatively high volatility of potassium salts (7, 8). To overcome this, solid ashing aids are added (7-9). However, the addition of solid ashing aids introduces the question of potassium contamination from the added reagents, an especially important consideration when very small amounts of potassium are being estimated. In electrodialysis, the apparatus and reagents are first cleaned by the passage of an electric current and thus a reagent blank is eliminated. The whole process takes place at low temperatures and thus loss of potassium is prevented.

On comparing the serum potassium values before and after electrodialysis, we were able to obtain evidence indicating that the precipitation of potassium as the cobaltinitrite is incomplete when applied directly to serum.

#### EXPERIMENTAL

*Estimation of Potassium before Electrodialysis*—This was carried out as described by Kaye (5), except that 0.2 ml. of solution was used to which 0.3 ml. of distilled water was added instead of starting with 0.5 ml. of solution. In the case of serum and recovery studies 2.0 ml. of 0.01 *N* ceric sulfate were employed to assure more than 70 per cent of excess oxidant. In the recovery studies 0.2 ml. of serum was measured out, to which 0.3 ml. of potassium chloride solution was added instead of distilled water containing a known amount of potassium. The empirical factor used in the calculations was redetermined at regular intervals and with each new batch of reagents.

*Estimation of Potassium after Electrodialysis*—The method and apparatus for electrodialyzing serum are essentially those described by Keys (10) and Sobel, Kraus, and Kramer (6) with some modifications in technique. An apparatus may be easily constructed with materials around the laboratory for twelve to twenty-four simultaneous electrodialysis units with the aid of an outside resistance.

The electrodialysis apparatus is set up with 5 ml. of doubly distilled water in the outer chamber and 5 ml. of approximately 0.01 *N* HCl (redistilled in an all-glass still and diluted with doubly distilled water) in the inner cathode chamber. The outer chamber consists of a tube containing a piece of sealed-in platinum wire. Mercury is placed in this tube so as to cover the bottom of the tube. The inner chamber is a tube open at both ends. At one end a piece of du Pont cellophane No. 300 is stretched

tightly and held in place with a rubber band. The other end is left open for an electrode. About  $\frac{1}{8}$  inch of mercury is placed on the cellophane (or more if necessary) to cover the tip of the electrode which is inserted into the cathode chamber. The electrode consists of a long, small bore glass tube with a sealed-in platinum wire.

In order to eliminate a blank value, a preliminary dialysis is run on 5 ml. of doubly distilled water. The dialysis is run for an hour, at the end of which time the inner tube is removed, washed three times with doubly distilled water, the mercury being shaken each time, and then refilled with 5 ml. of approximately 0.01 N HCl. The dialyzed water is left in the outer chamber to serve as a diluent in the subsequent electrodialysis. The liquid to be dialyzed was added to this chamber.

Dialysis was conducted on all solutions for about 15 minutes at maximum resistance of the rheostat in the circuit; then the resistance was cut to the minimum and the dialysis proceeded for 3 hours or more. At the end of the dialysis, the current was shut off and the inner chamber was removed. The mercury in it was shaken to break the mercury-base amalgam formed and the supernatant liquid was carefully aspirated into a 25 ml. Erlenmeyer flask. The chamber was then washed with 1 ml. of doubly distilled water and the washing aspirated into the flask. This was repeated four times, five washings in all. All the flasks were then placed in a 100–105° oven until dry. (The usual practice was to keep the flasks in the oven overnight.)

After cooling under a bell jar, doubly distilled water was added to each flask so as to make the volume of solution in the flask exactly equal to the volume of serum or known solution that was electrodialyzed for that particular flask. All solid material in each flask was thoroughly dissolved. 0.2 ml. of aliquot was used to which 0.3 ml. of distilled water was added and analyzed for potassium according to the Kaye method (5).

For the analysis of serum, 1.0 ml. of specimen was electrodialyzed and two 0.2 ml. portions analyzed for potassium. The rest of the dialysate was often employed for the analysis of sodium. In the comparative studies, 2.0 ml. of serum were electrodialyzed, 1 ml. of this being employed for the analysis for the presence of ammonia to rule out the possibility of a breakdown of protein into ammonia. The analysis for ammonia was carried out by a modification of the micro-Kjeldahl method (11, 12).

### *Results*

The values obtained in 67 determinations on inorganic solutions of potassium chloride containing 40.0  $\gamma$  of potassium, measuring 0.2 ml. of solution, are as follows: potassium found 40.6  $\gamma$ , average deviation  $\pm 0.82 \gamma$ , standard deviation  $\pm 0.93 \gamma$ . As shown above, good results are ob-

tained for known inorganic solutions, the average error being  $\pm 0.6 \gamma$  of potassium, which is 1.50 per cent of the total value, while the average deviation of the mean is 2.06 per cent of the total value, and the standard deviation is 2.48 per cent of the total value. These criteria indicate a good degree of precision and accuracy, considering the small amounts involved.

Recoveries in 51 determinations of known amounts (32.00  $\gamma$ ) of potassium added to serum when a sufficient excess of ceric sulfate was employed are 32.14  $\gamma$  for potassium, average deviation  $\pm 0.51 \gamma$ , standard deviation  $\pm 0.69 \gamma$ , values well within the error of the procedure.

Table I presents recoveries of potassium added to serum when only a slight excess of ceric sulfate is used in the titration. As may be readily observed, the values are less than those expected in the case of serum, while for inorganic solutions the values are within the experimental error

TABLE I

*Estimation of Potassium with Slight Excess of Ceric Sulfate*

1 ml. of ceric sulfate  $\approx$  69.5  $\gamma$  of K. The values are expressed in micrograms

Specimen	K found	K calculated*
Serum A	43.8	56.8
" B	52.0	57.4
" C	41.6	54.2
" D	50.8	53.6
Inorganic KCl Solution 1	60.0	60.0
" " " 2	60.0	60.0
" " " 3	59.6	60.0
" " " 4	59.2	60.0

\* Calculated as original serum K found + 25  $\gamma$  of K added.

of the method. Thus the need for a greater excess of ceric sulfate is demonstrated for the titration of the potassium cobaltinitrite precipitated from serum as compared to inorganic solutions.

The values obtained on the estimation before and after electro dialysis are presented in Table II.

As may be seen in Table II the mean values for serum potassium before electro dialysis are 10.4 per cent lower than those found after electro dialysis. This difference is statistically significant,  $P$  being approximately  $10^{-3}$  (13). In contrast to this difference the mean values of inorganic solutions as well as for known amounts of potassium added to serum are similar before and after electro dialysis.

A 1 ml. (representing 1 ml. of serum) portion of the electro dialysate in each of the above experiments was examined for the presence of ammonia to determine whether during the presence of electro dialysis some ammonia

is released. The presence of ammonia gives rise to higher values, since the ammonia precipitates as the sodium ammonium cobaltinitrite. All dialysates were found to be negative with respect to ammonia. The sensitivity of the micro-Kjeldahl method was tested by adding  $\text{NH}_3$  to a volume of indicator-boric acid solution such as would result from distillation. It was found that as little as 1.7  $\gamma$  of  $\text{NH}_3$  changed the indicator visibly. To ascertain further the ammonia interference, ammonia-potassium solutions were made up containing 2.55  $\gamma$  of  $\text{NH}_3$  and 35.0  $\gamma$  of potassium in absolute amounts. In six determinations the average potassium found was 34.7  $\gamma$  and the average deviation was  $\pm 0.43$   $\gamma$ . These

TABLE II

*Estimation of Potassium before and after Electrodialysis*

The values are expressed in micrograms.

Serum No.	K found in serum		Added K recovered from serum*		K found in KCl solution†	
	Before	After	Before	After	Before	After
1	32.0	35.7	34.0	34.3	34.4	35.0
2	32.4	36.7		36.0	35.0	35.0
3	32.2	34.3	36.6	34.9	35.1	34.7
4	32.7	39.1	34.6	35.5	34.6	34.3
5	37.0	41.2	37.8	34.8	35.6	34.9
6	29.0	33.6		35.2	35.1	35.4
7	68.1	69.5	34.8	34.6	35.8	34.5
8	34.1	41.3	34.4	34.5	35.2	35.0
Mean.....	37.1	41.4	35.4	35.0	35.1	34.9
P†.....	10 <sup>-8</sup> (approximate)					

\* 35  $\gamma$  added.

† 35  $\gamma$  present.

‡ P = probability that the difference between the means is due to chance.

results indicate that  $\text{NH}_3$  in small amounts does not interfere with the determination of potassium by the present method. Thus, this is additional evidence that the increased values following electrodialysis represent the potassium values rather than ammonia interferences.

#### DISCUSSION

In the electrodialysate employed for potassium analysis there is only a single anion present which depends upon the acid employed to trap the cation. This is of theoretical advantage in obtaining a cobaltinitrite precipitate of more constant composition which otherwise may be influenced by the variable mixture of anions, (such as sulfates, phosphates,

chlorides, carbonates) present in the ash of biological material obtained by the hitherto available methods. Furthermore, Harrison and Darrow (14) mention the desirability of removing phosphate for the estimation of the potassium as the potassium chloroplatinate because a gummy precipitate forms on the addition of alcohol which it is almost impossible to wash free of contaminants. When the potassium is determined as the potassium silver cobaltinitrite, the removal of chlorides is necessary (15). This may be accomplished by employing acetic or sulfuric instead of hydrochloric acid to trap the cations in the electro dialysis.

The method of electro dialysis is applicable in cases in which there are large amounts of ammonium ion present, *i.e.* urine. The ammonium ion may be removed according to Consolazio and Talbott (16) by a preliminary passage of electric current with only a platinum cathode without any membrane. Following this step the electro dialysate will be free of ammonium ions and may be employed for the estimation of potassium.

The completeness of electro dialysis has been demonstrated for red blood cells (16) and such electro dialysate could be advantageously employed for the estimation of potassium.

#### SUMMARY

Several advantages of electro dialysis as a preliminary step in the estimation of potassium have been demonstrated. As compared to ashing, loss of potassium due to volatilization is prevented. Contamination due to ashing aids is eliminated. Expensive quartz, nickel, or platinum vessels are not required. Truer values are obtained than when the method is applied directly to serum, the values being approximately 10 per cent higher following electro dialysis. This was not due to ammonia contamination, since the dialysate was free of ammonia. Furthermore, small amounts of ammonia did not interfere with the method employed in conjunction with electro dialysis. There is only one anion present in the electro dialysate, a fact that is at least of theoretical advantage in obtaining a cobaltinitrite precipitate of more constant composition. In this method the potassium cobaltinitrite precipitate was determined by the sensitive iodometric evaluation of the excess ceric sulfate which was used as the oxidant.

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## CARBONIC ANHYDRASE

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Carbonic anhydrase is an enzyme which catalyzes both phases of the reversible reaction  $\text{H}_2\text{CO}_3 \rightleftharpoons \text{CO}_2 + \text{H}_2\text{O}$ . The presence of this enzyme in mammalian red blood corpuscles was first demonstrated by Meldrum and Roughton (1) and independently by Stadie and O'Brien (2). Since chemical work on the enzyme has been reviewed in recent publications (3, 4) from these laboratories, we shall not deal here with the many valuable contributions which have been made by various workers in this field.

Carbonic anhydrase prepared by the methods used here is a light, white, amorphous hygroscopic powder containing approximately 8000 units of activity per mg. when tested according to the method already described (3). It is a protein having a nitrogen content of 15.9 per cent and a zinc content of 0.15 per cent (4). Adsorption, electrodialysis, or fractionation with inorganic salts and solvents did not result in any marked increase in the activity of this product. Consequently it seemed possible that the preparation was a pure, or almost pure, substance. Efforts were therefore made to prepare the enzyme in crystalline form. Early attempts at crystallization involved the use of such inorganic salts as ammonium sulfate, magnesium sulfate, and sodium sulfate. A wide range of acidities and temperatures was used but the precipitates which formed with the various salts were amorphous. There were, however, two properties of carbonic anhydrase which it was thought might be helpful in securing a crystalline preparation of the enzyme. In the first place this enzyme, as shown by Meldrum and Roughton (5) and confirmed by Scott and Mendive (4), is remarkably stable to alkaline solutions and, secondly, the enzyme contains zinc (6), a metal which forms complex salts with ammonia and certain other basic substances. It was accordingly decided to investigate the effect of ammonia in an alcoholic solution of the enzyme. However, the enzyme proved to be unstable in 40 per cent and insoluble in 60 per cent ethyl alcohol. By treating a concentrated solution of the enzyme with potassium dihydrogen phosphate, alcohol, and chloroform a product was obtained which was completely soluble in a slightly ammoniacal solution of 99 per cent ethyl alcohol. Moreover, there was no evidence of a decrease in potency of an alcoholic solution of the purified material during a period of 24 hours storage at room temperature if the diluted test solutions were allowed to stand for a few hours before the tests were made. It was thought

few drops of 5 N sulfuric acid were added and the material was heated and the ash content determined. The ash was dissolved in normal acetic acid, the solution transferred to a 15 cc. centrifuge thimble, and the acidity neutralized by the addition of ammonium hydroxide. The zinc content was then estimated by the method of Sahyun and Feldkamp (8). The ash content of the dried enzyme preparation was 0.3 per cent and the zinc content was 0.22 per cent. Potency determinations on an aliquot of the enzyme solution showed it to contain 10,000 units per mg. of solids. The nitrogen content was 15.8 per cent in terms of the solids.

*Experiment 2*—This experiment was very similar to Experiment 1. It was thought that perhaps a product might be obtained having a lower zinc content if a mixed phosphate buffer was used instead of dipotassium hydrogen phosphate. Accordingly a phosphate buffer solution was prepared by dissolving 0.6 gm. of sodium dihydrogen phosphate and 1 gm. of dipotassium hydrogen phosphate in 500 cc. of water. The pH of this solution was 7.1. To 0.5 gm. of the stock enzyme preparation were added 8 cc. of the phosphate buffer and 2 cc. of alcohol. The process of purification was then continued as in Experiment 1. By the evaporation of 14.0 cc. of enzyme solution after the dialysis against distilled water, the solid, ash, and zinc content was determined as before. The solids in the resultant enzyme preparation had an ash content of 0.32 per cent and contained 0.23 per cent zinc and 15.8 per cent nitrogen. The activity was 9800 units per mg. of solids.

In Experiment 3 a method is described for preparing carbonic anhydrase crystals by means of acetone and ammonium hydroxide. The ash, zinc, and nitrogen content and the activity of the preparation were determined. Experiments 4 and 5 provide additional data for the crystals.

*Experiment 3*—0.5 gm. of the stock enzyme preparation was first further purified by the method outlined in Experiment 1. After dialysis the aqueous enzyme solution was equally divided between two 250 cc. centrifuge thimbles. The solutions were dried *in vacuo*. To each thimble containing approximately 150 mg. of dried enzyme was added 1 cc. of N ammonium hydroxide. After the enzyme had dissolved, the tubes were placed in an ice bath. In another bath containing salt and ice was placed a flask containing 400 cc. of absolute acetone to which had been added 0.5 cc. of concentrated ammonium hydroxide. To each of the tubes containing the enzyme were added 200 cc. of chilled ammoniacal acetone and the tubes shaken. A marked sheen was obvious throughout the suspension in both tubes. The tubes were left in the ice bath for 5 hours. Examined microscopically the preparation appeared to be uniformly crystalline and to consist of very thin plates lying on one of their flat surfaces or, less frequently, on an edge. A photomicrograph of the crystals is shown in

Fig. 1. When the preparation was examined with polarized light between crossed nicols, those plates lying on a flat surface were isotropic, whereas those on an edge were anisotropic. After the 5 hour period the samples were centrifuged in the refrigerator and the crystals in each tube washed with 100 cc. of chilled acetone. After they were again centrifuged and the supernatant acetone decanted, the crystalline enzyme was dissolved in 25 cc. of water and this solution evaporated to dryness *in vacuo*. To the dried enzyme preparation 15 cc. of water were added. Some material remained undissolved and in suspension seemed to consist of fragments of thin plates which were isotropic. This material was removed by centrifugation and dried; it weighed about 15 mg. 14 cc. of solution were evaporated and the residue weighed and ashed by heating in the presence of a few drops of a concentrated solution of hydrogen peroxide. The ash content of the solids was found to be 0.8 per cent and the zinc content 0.14

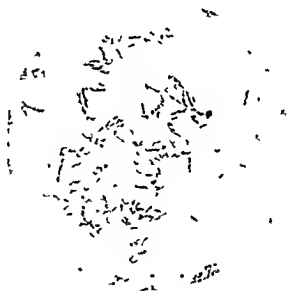


FIG. 1. Ammonium-carbonic anhydrase.  $\times 51$

per cent. The preparation had a potency of 8900 units per mg. and contained 16.5 per cent nitrogen.

*Experiment 4*—0.5 gm. of the stock enzyme preparation was further purified as in Experiment 2. After dialysis it was dried *in vacuo* in two 250 cc. centrifuge thimbles and crystallized with ammoniacal acetone as described in Experiment 3. The crystals were dissolved in 20 cc. of water and the excess acetone removed from the solution by allowing it to stand *in vacuo* overnight. 14 cc. of this solution were then evaporated and ashed as in Experiment 3. The ash content was found to be 0.3 per cent and the zinc content 0.21 per cent. The preparation had a potency of 8100 units per mg. and a nitrogen content of 16.5 per cent.

*Experiment 5*—It was realized in carrying out Experiments 3 and 4 that some of the ammonia might dissociate from the enzyme when the crystalline enzyme was dissolved in water and accordingly would be removed in drying *in vacuo*. Hence another experiment was conducted in which

60 mg. of the enzyme, purified according to the procedure used in Experiment 1, were crystallized according to the procedure outlined in Experiment 3. The crystals, after being washed with acetone, were dissolved in water and the nitrogen and solids determined on aliquot samples as before. The nitrogen content was 16.4 per cent.

For use in the following experiments a quantity of enzyme was prepared according to the procedure described in Experiment 1. After dialysis against distilled water had been completed, aliquots of the enzyme solution, containing the equivalent of about 10 mg. of dried enzyme, were placed in a series of 15 cc. centrifuge tubes. These solutions were then dried *in vacuo* and served as stock preparations in the following experiments. Before the various reagents were used, they were carefully distilled.



FIG. 2

FIG. 2. Piperidine-carbonic anhydride.  $\times 20$ .

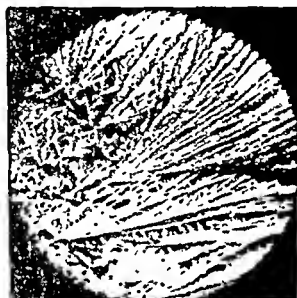


FIG. 3

FIG. 3. Isoamylamine-carbonic anhydride.  $\times 23$ .

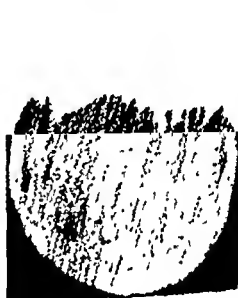


FIG. 4

FIG. 4. *n*-Amylamine-carbonic anhydride.  $\times 20$ .

*Piperidine Experiments*—To each of six tubes containing about 10 mg. of enzyme 0.5 cc. of water was added. After the enzyme had dissolved, the tubes were immersed in a cold water bath and 1 cc. of piperidine slowly added to each. The contents of four tubes were then poured on four watch-glasses and the solutions allowed to evaporate at room temperature. In about 3 hours the liquid had evaporated, leaving a crystalline preparation having a sheaf-like appearance. The watch-glasses were placed *in vacuo* over calcium chloride or sulfuric acid and flake sodium hydroxide for 48 hours. A photomicrograph of the crystals is shown in Fig. 2. The crystals were anisotropic. The material from three of the glasses was then used for Dumas nitrogen determinations. The crystals were found to contain 16.1 per cent nitrogen. The material on the fourth glass was removed by washing with 100 cc. of 0.05 per cent peptone. The product was insoluble. The potency of a diluted sample of the suspension was

estimated immediately and again after it had stood for 24 hours. The contents of the fifth tube, after standing for 15 minutes at room temperature, were suitably diluted with peptone solution (0.05 per cent) at 0° and a potency estimation made in the usual manner. The diluted enzyme solution was then left at room temperature for 24 hours and the potency again determined. The sixth tube containing the enzyme and piperidine was allowed to stand for 3 hours at room temperature. The solution was

TABLE I  
*Potency Tests of Carbonic Anhydrase Preparations*

Preparation tested	Immediate test	Potency	Test after 24 hrs.	Potency
	<i>total units</i>	<i>per cent of control</i>	<i>total units</i>	<i>per cent of control</i>
Control enzyme.....	92,000		92,000	
Piperidine + carbonic anhydrase standing 15 min.....	11,700	12.7	97,000	105
Piperidine + carbonic anhydrase standing 3 hrs.....	3,750	4.1	31,000	33.7
Piperidine-carbonic anhydrase crystals (dried).....	1,176	1.3	1,528	1.6
Control enzyme.....	92,000		92,000	
Isoamylamine + carbonic anhydrase standing 15 min.....	11,400	12.4	82,200	90.0
Isoamylamine + carbonic anhydrase standing 2 hrs.....	600	0.7	15,000	16.3
Isoamylamine-carbonic anhydrase crystals (dried).....	0	0	0	0
Control enzyme.....	92,000		92,000	
<i>n</i> -Amylamine + carbonic anhydrase standing 15 min.....	5,100	5.5	75,300	81.9
<i>n</i> -Amylamine + carbonic anhydrase standing 3 hrs.....	300	0.3	7,500	8.2
<i>n</i> -Amylamine-carbonic anhydrase crystals (dried).....	16	0	128	0.1

then diluted with peptone at 0° and a potency estimation made. The diluted test solution then stood for 24 hours at room temperature and the potency was again determined. The results of various potency determinations are shown in Table I.

*Isoamylamine Experiments*—These experiments were carried out in a manner which was very similar to the experiments with piperidine outlined above. To each of six tubes containing approximately 10 mg. of enzyme was added 0.5 cc. of water. After the enzyme had dissolved, the tubes

were immersed in a cold water bath and 1 cc. of isoamylamine added to each. The contents of four tubes were poured on four watch-glasses and left at room temperature. In 2 hours the material had dried, leaving a needle-like crystalline product. Drying was continued *in vacuo* in a manner similar to that used in the piperidine experiments. A photomicrograph of the crystals is shown in Fig. 3. The crystals were anisotropic. The crystalline precipitates from three of the glasses were used for Kjeldahl nitrogen estimations. The nitrogen content was found to be 16.2 per cent. The material on the fourth watch-glass was suspended in dilute peptone solution and used for potency estimations. The solution in the fifth tube, after standing for 15 minutes, was suitably diluted with 0.05 per cent peptone at 0°. The diluted solution was allowed to stand for 24 hours at room temperature and again assayed. The sixth tube stood for 2 hours at room temperature after the addition of the isoamylamine. Then the solution was diluted and potency tests made immediately and in 24 hours time. The results of these potency tests are shown in Table I.

*n-Amylamine Experiments*—*n*-Amylamine has a boiling point approximately that of piperidine. Consequently, the process used for the crystallization of carbonic anhydrase with this amine was the same as that already outlined for the piperidine experiments. After the crystalline preparation had been dried *in vacuo* for 48 hours, a photomicrograph was taken and is shown in Fig. 4. The crystals were anisotropic. The preparation had a nitrogen content of 16.1 per cent. The results of activity experiments are recorded in Table I.

#### DISCUSSION

Two general methods are described for producing crystalline preparations of carbonic anhydrase. In the first set of experiments crystals were obtained from dilute suspensions of the enzyme in acetone containing ammonium hydroxide. The crystals were readily soluble in water and exhibited only slightly less activity than the non-crystalline enzyme from which they were prepared. This difference in potency may indicate a slight inactivation of the enzyme during crystallization or that the ammonium-carbonic anhydrase complex does not completely dissociate in water. It should be noted, however, that if the crystals were dried they were no longer soluble and did not exhibit activity. The zinc content of the preparation was not changed by crystallization. The nitrogen content of the crystalline material was 16.5 per cent, whereas that of the non-crystalline product was 15.8 per cent. It seems evident, therefore, that the ratio of enzyme to ammonia is very high.

The second set of experiments was concerned with compositions of carbonic anhydrase and the liquid bases piperidine, isoamylamine, and

*n*-amylamine. From potency results recorded in Table I, it can be seen that when solutions of the enzyme and the various bases were allowed to stand for 15 minutes and then immediately tested there appeared to be a very substantial loss in activity. However, if these diluted test solutions were allowed to stand for 24 hours, practically all the potency was recovered. When the preparations stood for 2 or 3 hours, a similar apparent inactivation and a recovery of potency on standing was apparent. In these latter experiments, however, a much lower recovery was observed. This probably means that the base has become more firmly bound to the enzyme on standing and accordingly requires more rigorous treatment for its removal rather than that any permanent inactivation of the enzyme has occurred. In respect to some of the potency tests it will be noted that dilutions were made at 0°. It was thought that the dissociation between the base and the enzyme would be less at this temperature than at a higher temperature. The actual test was, of course, conducted at 15°. From Table I it is also apparent that all the crystalline preparations formed by carbonic anhydrase and these liquid bases were inactive when dried and did not regain activity when suspended in water. This is not surprising, for the crystalline preparation of ammonia-carbonic anhydrase also loses its activity, as do some other crystalline enzyme preparations, when dried. All the results in Table I support the view that a chemical combination has taken place between the enzyme and the bases and that the crystals are the product of this combination. Evaporation of control solutions of enzyme did not result in the formation of crystals. The nitrogen values obtained for the three crystalline preparations are only slightly higher than that determined for the non-crystalline enzyme. This might be expected, since the percentage of nitrogen in each of the bases used does not differ greatly from that of the protein. The dried enzyme-base crystals were all anisotropic. Each of the three crystalline preparations was heated for 1 hour at 110° and again examined microscopically by transmitted light and between crossed nicols. No change in crystalline nature was observed.

The success of obtaining crystalline compounds of the enzyme with piperidine, isoamylamine, or *n*-amylamine probably depends on many factors. In the crystallization experiments the proportion used was 2 parts of base to 1 part of water. When such a solution containing no enzyme is allowed to evaporate at room temperature, crystals form as evaporation progresses. These crystals are soluble in water and disappear at 110° or *in vacuo*. This property of forming crystals probably initiates crystallization in the experiments of the enzyme-base complex. In the experimental part of the work a control solution of water and base was always evaporated at the same time as similar solutions to which enzyme had been added. When these control samples were dried *in vacuo* for 12 hours, no residue remained.



The crystalline enzyme preparations were always dried for 48 hours. A second factor which may be important in securing crystalline preparations in the present experiments is the fact that the bases used had a boiling point near that of water. In experiments with carbonic anhydrase and *n*-butylamine, an amine boiling at approximately 78°, only a very poor yield of crystals was obtained. It seems probable that the amine evaporated too quickly during the process. The results of experiments conducted with ethylenediamine, an amine boiling at 118°, were not completely satisfactory.

Experiments were conducted to determine the possibility of precipitating carbonic anhydrase from aqueous solution by the addition of basic substances. When undecylamine, an amine used by two of our colleagues to precipitate proteins (9), was added to a solution of the enzyme, no precipitate formed. Protamine likewise did not precipitate the enzyme from an aqueous solution. It was of interest to apply the methods of crystallization outlined in this paper to other proteins and also to other substances having free acidic groups. When either piperidine, isoamylamine, or *n*-amylamine was used with insulin or heparin in a manner similar to that described in the experiments with carbonic anhydrase, crystalline products were obtained. These products, however, differed in certain respects from the dried crystalline enzyme preparations, since both the crystalline preparations of insulin and of heparin were soluble in water and contained physiological activity. The fact that heparin, which contains free sulfate radicals, crystallized with these substances is of interest. The *n*-amylamine-carbonic anhydrase and *n*-amylamine-insulin crystals appear as long needles, whereas the *n*-amylamine-heparin crystals occur as rosettes resembling in appearance those previously isolated as the barium salt (10). Thus the shape of crystals would not appear to be dependent solely upon the bases used to effect crystallization. Sedimentation, diffusion, and electrophoresis analyses of the carbonic anhydrase are being made in the Department of Chemistry at the University of Wisconsin and will be published shortly by M. L. Petermann and N. V. Hakala.

#### SUMMARY

Methods are described for the further purification of carbonic anhydrase. The resultant preparations contained 0.3 per cent ash, 0.2 per cent zinc, 15.8 per cent nitrogen, and had an activity of 10,000 units per mg. A method of producing a crystalline preparation of the enzyme by means of acetone and ammonium hydroxide is described. The crystals were active prior to drying. They contained 16.5 per cent nitrogen and 0.2 per cent zinc. Other methods of obtaining crystalline preparations of the enzyme by the use of piperidine, isoamylamine, and *n*-amylamine are also described.

These preparations when dried were insoluble and inactive. The three crystalline preparations were anisotropic. After the crystalline enzyme preparations were heated for 1 hour at  $110^{\circ}$ , no change in the shape of the crystals was evident with either transmitted light or between crossed nicols. Various aspects of the research are discussed.

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## THE BIOCHEMISTRY OF STRONTIUM\*

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Since the observation by Mendel and Thaeher (1) in 1904 that strontium was excreted in part by way of the intestine by the dog, there have been numerous investigations of the physiological effects of this element. Most of these studies were limited in scope for lack of an adequate quantitative method for strontium. Several investigators ignored the similarity in chemical behavior of strontium and calcium and reported that strontium administration increased the level of serum calcium. It was suggested that strontium increased the permeability of the intestine to calcium (2). One of us (3) pointed out that such values represent the sum of the strontium and calcium in the blood, since they are precipitated together in the usual oxalate precipitation. The publication by Sobel and his collaborators of a method to determine small amounts of strontium in the blood stimulated the present study. We have investigated the level and state of calcium, strontium, and inorganic phosphate in the blood following the administration of small amounts of strontium, after long continued administration and after intravenous injection. The excretion of these ions in urine and feces was followed. A few determinations of magnesium were included in the blood studies.

### *Methods*

Calcium and strontium were determined by the Sobel method (4), which is based upon the fact that, if oxalate and sulfate be added to a mixture of calcium and strontium at a pH of 3.0, the calcium is selectively precipitated as oxalate and the strontium as sulfate. Calcium and strontium are precipitated as oxalate under controlled conditions and strontium is calculated by difference. There are disadvantages in any determination by difference, particularly if small amounts of one substance be determined in the presence of large amounts of the other. We found the greatest error in the method in the determination of strontium in feces. When 7.4 mg. of stron-

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<sup>‡</sup> Now at the Henry Ford Hospital, Detroit.

tium were added to a sample of feces containing 20.8 mg. of calcium, the error was -8.1 per cent. The small quantity and the large conversion factor (determined by the higher molecular weight) magnify the error for strontium. Repeated determinations on solutions and on serum and urine gave recoveries sufficiently accurate to warrant experimental use. Analyses were made in triplicate when possible, otherwise in duplicate.

Protein-free serum filtrates for the determination of filtrable fractions were prepared by the Gregory and Andersch modification (5) of the Greenberg and Gunther method (6). The Fiske and Subbarow method (7) as modified for the Evelyn photoelectric colorimeter was used for inorganic phosphate and the Briggs method (8) for magnesium. Proteins were determined by the Robinson, Price, and Hogden procedure (9).

Adult female dogs maintained at constant weight on a Cowgill diet (10) were used in this study. Urine collections were begun and ended by catheterization and feces were collected in the usual manner with carmine as the marker. Strontium was given as the lactate.

### *Results*

Table I gives the results of two representative experiments showing the effect of single doses of strontium given by mouth upon the calcium, strontium, and inorganic phosphate of the serum. These with many similar experiments show that administration of 20 to 192 mg. of strontium per kilo of body weight usually produces a fall in serum calcium which may occur several days after the strontium is given and which does not necessarily coincide with the highest level of strontium. An increase of inorganic phosphate in the serum was produced which bore no quantitative relation to the height of the calcium and strontium. Greenberg and Gunther (11) report a rise in serum phosphate 6 hours after the ingestion of calcium which we found to be true also for strontium. Both the time of appearance and the level of strontium in serum varied widely after ingestion. Figures for magnesium are omitted from our tables as the response was neither striking nor consistent.

In order to eliminate the effects of delay in or failure of absorption, strontium was given by the intravenous route. We injected amounts of strontium which varied from 4.43 to 100 mg. per kilo of body weight. Except for nausea in one animal no toxic symptoms resulted from doses up to 28.4 mg. per kilo, the molar equivalent of the 13 mg. of calcium reported by Cowgill and Rakieten (12) to be the maximum amount tolerated by their dogs without nausea. Extreme salivation, nausea, and diarrhea followed the injection of 100 mg. of strontium per kilo of body weight. The height of the serum strontium varied from 1.5 to 51.9 mg. per 100 ml. following the injection, depending upon the amount given and

the time at which the blood was taken. Strontium disappeared rapidly from the blood after injection, reaching zero within 24 hours in all cases studied. A decrease in serum calcium appeared rapidly after the injection and persisted for 24 hours or longer. The immediate effect upon phosphate was variable but an increase after 5 to 6 hours occurred regularly. Control injections of calcium produced a prompt decrease in inorganic phosphate. Table I gives the results of one injection experiment.

TABLE I

*Effects of Single Doses of Strontium Lactate on Serum Composition*

The values are expressed as mg. per 100 ml. of serum.

Day No.	Serum			Remarks
	Ca	Sr	P	
1	11.90		5.36	Dog M, control Fed 20.2 mg. strontium per kilo body weight
2	9.05	0.44	5.79	
4	9.79	0.56	6.04	
6	11.80	1.43	4.15	
8	11.81	0.22		
15	11.23	0.0	4.87	
	9.32	0.0	(5.4)	Dog M, control; phosphate average before control day Fed 192 mg. strontium per kilo body weight Vomited; slight diarrhea
1				
2	8.6	0.84	5.43	
4	9.6	0.30	5.68	
7	10.51	0.0	5.15	
1. 10.30 a.m.	10.7	0.0		Dog W given intravenously 33.6 ml. 2.2% strontium lactate (8.5 mg. strontium per kilo), pH 7.1, 11.07-11.19 a.m.
1. 11.25 "	9.8	7.1	3.5	
2	7.6	0.0	3.97	
3	9.6	0.0	4.25	

In Tables II and III are summarized analyses for total and filtrable strontium and calcium of the serum after strontium administration. The filtrable strontium varies from 0 to 83 per cent of the total and bears no constant relationship to the total level or to the level of total or filtrable phosphate.

Several animals were fed 20 mg. of strontium per kilo of body weight per day for periods of 20 to 140 days without toxic symptoms. Table III

shows figures for two such experiments. There are wide variations in the levels of calcium and strontium in the blood and there is no constant inverse relation between them. The inorganic phosphate rose slightly during the period of administration and returned to the normal level when strontium was no longer given. Strontium was found in the blood for some weeks after the dosage had been discontinued. The samples of leg bones which were analyzed contained small amounts of strontium.

TABLE II

*Filtrable Calcium and Strontium in Serum after Strontium Administration*

The calcium, strontium, and phosphorus values are expressed as mg. per 100 ml. of serum.

Dog	Serum						Serum protein gm.	Blood pH	Remarks
	Ca		Sr		P				
	Total	Filtrable	Total	Filtrable	Total	Filtrable			
V	9.41	4.24	4.62	3.15	4.9		7.42	16 hrs. after feeding 1.34 gm. strontium, 100 mg. per kilo	
M	9.8	3.96	1.58	1.31	6.6		7.34	5 hrs. after 1.53 gm. strontium by stomach tube, 70.2 mg. per kilo	
L	13.2	7.92	16.2	0.0	4.5	1.9	6.92	7.52	3 min. after injection of 28.4 mg. per kilo strontium
W	9.8	6.7	7.1	1.23	3.5		6.92	7.36	6 min after injection of 8.5 mg. per kilo strontium
A1	7.2	5.0	7.0	3.01	2.3	2.1	5.7	7.34	12 min. after injection of 6.27 mg. per kilo strontium
An	12.9	7.8	8.5	1.03	4.31	3.4		7.34	15 min. after injection of 28.4 mg. per kilo strontium
A2	10.8	6.42	1.54	0.0	3.19	2.69	6.0	7.42	2 hrs. after injection of 4.43 mg. per kilo strontium

All filtrable values have been recalculated for serum. When protein was not determined, the values were divided by 1.06.

The excretion of strontium was studied (a) after one dose by mouth, (b) after intravenous injection, and (c) after long continued administration by mouth. Table IV gives representative experiments in which a single dose of strontium was given by mouth or by intravenous injection. It is obvious that after administration by mouth the largest part of the strontium is excreted by way of the feces and that after injection the largest part is found in the urine.

In the long experiments, ranging from 20 to 40 or more days, the total amount of strontium excreted was found to be 28 to 87 per cent of that fed. Of this 88 to 96 per cent was excreted in the feces. During the 1st week of such an experiment the excreted strontium might amount to as little as 13 per cent of the total given with 68 per cent of the excreted material

TABLE III  
*Effects of Long Continued Administration of Strontium Lactate*

Day No.	Serum								Remarks
	Ca		Sr		P	Protein			
	Total	Filtrable	Total	Filtrable		Total	Albumin	Globulin	
	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	gm. per 100 ml.	gm. per 100 ml.	gm. per 100 ml.	
1	10.00	5.84	0.0	0.0	4.32	6.37	3.42	2.95	Dog W, control; 320 mg. (20 mg. per kilo) strontium per day begun in food
15	9.67	3.74	2.26	0.1	5.24	6.8	3.15	3.65	
23	8.59	5.95	0.44	0.0	5.69				
72	9.08	5.14	2.64	1.78					
99	10.63	5.37	?	0.0	3.45	6.34	3.10	3.24	
120	7.55	4.37	3.63	2.62	5.04				Strontium discontinued
141	8.43	4.70	0.92	0.31	4.93	6.15	3.23	2.92	
162	8.90	3.49	0.99	0.0	4.52	6.11	3.23	2.88	" disappeared from blood 47 days after discontinuance
1	10.60	5.76	0.0	0.0		6.06	3.15	2.91	Dog J, control; 263 mg. (20 mg. per kilo) strontium per day begun in food
15	9.91	5.46	3.30	0.0		6.50	3.58	2.92	
50	8.41	6.15	3.38	0.62					
99	9.94	6.04	1.32	0.0	3.65	6.05	3.87	2.18	Appetite poor
143	10.80	5.02	2.06	1.07	4.70	6.58	3.68	2.90	Animal killed; gross pathological examination showed normal tissues; * analysis of dried portion of femur gave Ca 25.6, Sr 2.95%

\* Examination of the intestinal and urinary tracts, of the liver, heart, and lungs was made for us by Dr. Mollie Geiss, to whom our thanks are due.

in the feces; but continuation of the dose resulted in larger excretions, particularly in the feces. This may be due to decreased absorption. When strontium is discontinued, it invariably disappears from the feces much more rapidly than from the urine. Thus in one experiment it was not found in the feces after the 2nd week, while it persisted in the urine for more than 8 weeks. This point is illustrated in Table V, which also gives



a detailed summary of the effect of strontium upon the excretion of calcium and phosphate.

TABLE IV  
*Excretion of Strontium after Single Dose of Strontium Lactate*

Days	Strontium								Remarks
	Urine		Feces		Excreted		Per cent of excreted		
	Total		Total		Total		Urine	Feces	
	mg.	per cent	mg.	per cent	mg.	per cent			
1	5.0	0.18	550	20.2	556	20.3	0.90	99.20	Dog M, 2.74 gm. (126 mg. per kilo) Sr by mouth. Feces and urine 6 hrs. later; slight diarrhea
8	39.0		280						
	44.0	1.60	830	30.3	876	32.0	5.02	94.98	Total excretion in 9 days
1	7.9								Dog W, 0.203 gm. (8.5 mg. per kilo) Sr by intravenous injection. Urine 2 hrs. later
6	21.1	14.3	0	0.0	29	14.3	100.0	0.0	Total excretion in 6 days

TABLE V  
*Average Excretion per Day of Strontium, Calcium, and Inorganic Phosphorus*

Days	Urine			Feces			Remarks
	Ca	Sr	P	Ca	Sr	P	
	mg.	mg.	mg.	mg.	mg.	mg.	
28	29.9	21.4	480	517	175	599	Dog W, fed 0.32 gm. Sr (20 mg. per kilo) per day, in basal diet
16	No analyses made						
42	17.2	22.0	511	625	203	477	
5	No analyses made						
21	14.4	18.6	596	433	132	368	Strontium discontinued
7	29.6	2.4	775	473	55	312	
7	61.2	53.0	820	357	21	372	
7	19.6	3.7	664	776	0	447	
7	26.7	6.4	755	398	0	286	
7	11.6	7.1	655	307	0	364	
35	29.7		734	462		356	Average for 5 wks.

#### DISCUSSION

Strontium given intravenously or by mouth in large or small doses produces a fall in serum calcium. This decrease, whether immediate or

delayed, does not always accompany the highest values for serum strontium and may be in the diffusible or in the non-diffusible fraction. The experiments given in Table III illustrate the lack of correlation between reduction of the total calcium and the height of the diffusible calcium and also show that there is no constant effect upon calcium as a result of high or low strontium levels.

After injection strontium may appear in both filtrable and non-filtrable forms, differing from calcium which is reported to increase almost completely in the diffusible fraction after administration (11). Our experiments suggest that upon injection of strontium a non-dialyzable strontium phosphate compound is formed which disappears rapidly, the phosphate returning to the normal filtrable form (13) and some of the strontium becoming filtrable. The amount of non-filtrable phosphate is not always great enough to account for the strontium, which suggests the possibility of a strontium-protein combination. Smith and Sternberger (14) and others have inferred that the protein in the serum is not saturated with calcium under ordinary conditions. The highest value for non-diffusible strontium which we obtained was 16.2 mg. (Dog L, Table II). If tertiary strontium phosphate be the compound formed (the tertiary salt is supposed to be formed after calcium injections (13)), the 2.6 mg. of non-diffusible phosphate could combine with 11 mg. of strontium, leaving 5.2 mg. to combine with protein, not an excessive amount in the light of the calcium figures reported by Smith and Sternberger. Our other experiments show lower figures for non-diffusible strontium but only in one case (Dog A2, Table II) is there sufficient non-diffusible phosphate to account for the strontium and in that experiment the total strontium was low. It would be interesting to determine the distribution ratio between calcium, strontium, and protein by means of the procedure of Loeb (15) and we have made a few preliminary experiments of this kind.

The inorganic phosphate of the serum behaved after strontium administration in much the same way that it does after calcium injections. It rose within 5 to 6 hours and on long continued administration the level tended to be higher than normal and decreased when strontium administration ended. The formation of non-filtrable phosphate after injection of strontium likewise resembled the effect of calcium (13).

A few determinations of magnesium which we have made indicate that in the blood the level of this element is independent of calcium, strontium, and inorganic phosphate as suggested by the experiments of Tibbetts and Aub (16).

The figures for diffusible and non-diffusible calcium, strontium, and inorganic phosphate and for serum proteins show no apparent relationship to each other when various equations are applied (17, 18). Strontium is

deposited in the bones and other tissues (19).<sup>1</sup> When we consider that an equilibrium would have to be established not only in the blood but also in the tissue fluids and bones and that strontium is excreted in urine and feces, it is not surprising that such a complex picture cannot be described by any formula involving only the blood levels.

The pH of the blood following strontium injections showed no abnormal values, although one of 7.52 (Dog L, Table II) might be considered high. This is well within the range of pH 7 to 8 in which calcium distribution between the two fractions is reported to be constant (13), although Dillman and Visscher (20) disagree with this idea. This high figure occurred in the experiment in which the greatest amount of non-filtrable strontium was found and might bear out the statement of these authors that at pH 7.6 there is less filtrable calcium than at pH 7.0.

The studies on excretion show that like calcium (21) strontium when given by mouth is excreted in large part in the feces. When given intravenously in small amounts, strontium appears in the urine and may be absent from the feces, again resembling calcium (22, 23). McCance and Widdowson (24) using a spectrographic method of analysis found a slight increase in fecal strontium after intravenous injection of small quantities in man. The large amount of strontium found in the feces after oral administration is in great contrast to the small amount following intravenous injection (Table IV) and suggests that failure in absorption may be responsible in large part for the fecal strontium after oral administration. Small amounts may be excreted, like calcium, in the digestive juices and in bile after intravenous administration and there is no necessity to invoke any specific excretory function of the intestinal epithelium for strontium under normal conditions.

The effect of strontium on the excretion of calcium and phosphorus is complicated by a number of factors. With a controlled diet under fairly rigid conditions strontium produced a decrease of calcium and phosphate in the urine and an increased calcium in the feces (Table V). The phosphate of the feces changed very little. Upon withdrawal of strontium the calcium did not change in the urine and decreased in the feces, while the level of phosphate increased in the urine and decreased in the feces. Any explanation of these findings must take into account the deposition in bone and the distribution in other tissues, the calcium and phosphorus balance of the animal throughout a long period, and various other factors which we are not prepared to discuss at this time. We can state that for adult dogs the addition of strontium to the diet does not produce a marked

<sup>1</sup> Fay, M., Behrmann, V. G., Andersch, M. A., and Geiss, M., unpublished data.

excretion of calcium and does encourage a retention of phosphate, probably to combine with the strontium deposited in bone. This does not apply to young growing animals, as studies in which the strontium metabolism was contrasted in adult and in young rats have shown.<sup>1</sup>

#### SUMMARY

Filtrable and non-filtrable strontium, calcium, and inorganic phosphorus of the serum were determined in adult dogs after administration of strontium by mouth or by intravenous injection in large or small doses and after long continued feeding. Strontium may exist in the blood in diffusible form and probably combines with phosphate and with protein in non-diffusible compounds. Serum calcium is decreased after strontium is given and the decrease cannot be ascribed to a simple replacement effect, since there is no constant inverse relation of strontium and calcium levels. The inorganic phosphate of the serum showed a rise 5 to 6 hours after the administration of strontium and tended to remain high during periods of long continued feeding of strontium. No effect upon serum protein levels was observed. A few magnesium determinations suggested that magnesium acts independently of strontium.

The studies on excretion showed that strontium given by mouth is excreted in large part in the feces and when injected intravenously may be absent from the feces and appear in the urine. Small amounts disappear rapidly from the blood after injection but are not excreted completely, as much as 97 per cent remaining in the body after a week. Analyses of bone showed that strontium had been deposited there. Strontium in the diet of adult dogs did not produce a marked excretion of calcium but increased the retention of phosphate.

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# PANTOTHENIC ACID IN THE METABOLISM OF *PROTEUS MORGANII*\*

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Although several members of the vitamin B complex are now known to serve as coenzymes in certain essential metabolic reactions, the physiological function of pantothenic acid is obscure. The only evidence relating pantothenic acid to metabolism is the report of Pratt and Williams (1) that pantothenic acid stimulated fermentation and respiration of yeast (sucrose as substrate), and respiration of apple and potato tissue, with indications of a similar effect on certain animal tissues. The meaning of the effects on yeast is not clear, since the authors record multiplication of the yeast under the conditions of their experiments. Stimulation of the respiration of the potato and apple tissues was small, and in a number of the experiments appeared to be within the range of experimental error.

Studies in this laboratory were undertaken to determine the manner in which pantothenic acid is concerned in metabolic reactions and this report deals with the results of such studies on the metabolism of one of the bacteria. It was found that pantothenic acid is involved in the metabolism of pyruvic acid by *Proteus morganii*.

Preliminary work involved the trial of bacteria known to require pantothenic acid for growth. Our first experiments were undertaken with certain members of the *Pasteurella* group, as previous work (2) had shown that calcium pantothenate is needed by these organisms. However, cultures of the organisms produced a considerable amount of gum which led to difficulties in the centrifuging and the obtaining of suitable suspensions of cells. Irregularity of results led us to use instead *Proteus morganii*, which Pelczar and Porter (3) have shown requires nicotinic acid and calcium pantothenate for growth. All data presented in this report were obtained by the use of *Proteus morganii*.

## Methods

**Bacterial Suspensions**—For our experiments *Proteus morganii* was grown on a semisynthetic medium containing 0.5 per cent hydrolyzed Eastman

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purified gelatin, 0.5 per cent NaCl, 0.2 per cent  $K_2HPO_4$ , 0.005 per cent  $MgSO_4$ , 0.001 per cent  $CaCl_2$ , and 0.002 per cent cystine. The pH was adjusted to 7.0. Nicotinamide was added in the amount of 0.1  $\gamma$  per cc. and calcium pantothenate was added in a concentration of 0.0003  $\gamma$  per cc. unless otherwise specified.

Inoculations were made into liter flasks containing 800 cc. of medium and the flasks were incubated for 24 hours at 37°. The cells were collected by centrifugation and were washed twice with and suspended in 0.05 M phosphate buffer (pH 7.4).

*Metabolic Experiments*—Oxygen uptake was measured by the direct Warburg method. Respiratory quotient was determined by the method of Warburg (4).

*Analytical Methods*—Pyruvate was determined by the method of Lipton.<sup>1</sup> The respiration was stopped in the pyruvate experiments by the addition of 2 cc. of 0.1 N acetate buffer (pH 4.5). Samples were taken directly for pyruvate determinations.

Acetate was determined by a modification of the lanthanum nitrate reaction (5). The test was performed as follows: To the reaction mixture were added 2 cc. of 10 per cent  $H_2SO_4$  and the sample was slowly distilled, the distillate being collected in several fractions. 0.5 cc. of 0.1 N  $I_2$  in KI and 0.5 cc. of 5 per cent lanthanum nitrate were mixed in a test-tube and 0.2 cc. of the unknown sample was floated on the surface. On top of this was floated 0.5 cc. of N ammonium hydroxide and the mixture carefully placed in a boiling water bath. In the presence of 1:5000 acetate a blue-green precipitate forms at the interface. Distillation is necessary, since both lactic and pyruvic acids interfere with this test.

#### EXPERIMENTAL

The principle involved in these experiments was to grow cells on a medium deficient in calcium pantothenate and to study the effect of added calcium pantothenate on the metabolism of such cells. The amount of calcium pantothenate used in the culture medium did not permit optimum growth, but was sufficient to permit harvesting of a crop of cells. Our first experiments with *Pasteurella* causing hemorrhagic septicemia indicated that calcium pantothenate stimulated oxidation of glucose only slightly. The results consistently showed some stimulation but the magnitude was variable and at times was so small as to cast doubt on the conclusions. Experiments with *Proteus morganii* showed that calcium pantothenate consistently stimulated oxygen uptake with glucose as substrate. However, when lactate was substituted for glucose a much greater stimulation was obtained.

<sup>1</sup> Lipton, M. A., personal communication.

Table I gives the results of two typical experiments with lactate as substrate. In the first experiment (Experiment A) cells were grown on a medium containing an excess of calcium pantothenate (0.1  $\gamma$  per cc.). Such cells show no stimulation of respiration on the addition of calcium pantothenate to the washed suspension. In contrast, the addition of calcium pantothenate to cells grown on a deficient medium (0.0003  $\gamma$  per cc.) results in a marked stimulation of respiration (Experiment B).

In view of the previous report of Pratt and Williams (1) experiments

TABLE I

*Relationship of Respiratory Stimulation by Calcium Pantothenate to Calcium Pantothenate Content of Culture Medium*

0.3 cc. of 2 per cent lactate was added to 2 cc. of cell suspension and the final volume was made up to 2.7 cc. in every case. 0.1 cc. of 20 per cent KOH was placed in the center cup.

	Experiment A Cells grown on 0.1 $\gamma$ per cc Ca pantothenate		Experiment B Cells grown on 0.0003 $\gamma$ per cc Ca pantothenate	
	O <sub>2</sub> in 2 hrs	Stimulation	O <sub>2</sub> in 2 hrs.	Stimulation
	c mm	per cent	c mm	per cent
Cell suspension	479.8		247.2	
" " + 20 $\gamma$ Ca pantothenate	477.2	0	545.2	120

TABLE II

*Cell Counts in Presence and Absence of Calcium Pantothenate in Warburg Vessels Lactate used as substrate.*

	O <sub>2</sub> in 2 hrs	Stimulation	Cell count at end of experiment
	c mm	per cent	$\times 10^3$
Cell suspension	159.4		3.4
" " + 20 $\gamma$ Ca pantothenate.	387.9	137.5	3.5
" " .	247.2		5.7
" " + 20 $\gamma$ Ca pantothenate..	545.3	120	6.5

were performed in which sucrose was used as substrate. Sucrose was oxidized only slightly if at all and no effect of calcium pantothenate was observed.

In the absence of added substrate the respiration was very low and showed little if any stimulation by pantothenic acid.

The experiments in Table II were designed to determine whether the stimulation was due to an actual stimulation of respiration or whether it was merely due to an increase in the number of cells. While multiplication was hardly to be expected under these conditions, since the suspending



The results presented above indicate that lactic acid is being oxidized through at least two steps; that is, the lactic acid is first oxidized to pyruvic acid and the latter compound is then oxidized further, possibly to acetic acid.

Since pantothenic acid stimulates both the oxidation of lactic and pyruvic acids, it can be concluded that the site of action of this compound is either solely on the oxidation of pyruvic acid, or on both the oxidation of lactic acid to pyruvic acid and the further oxidation of pyruvic acid. The next experiments were undertaken to obtain further information with respect to these two possibilities.

The use of ketone fixatives to prevent the second reaction was first tried but was of no help, since all of the ketone fixatives used inhibited both reactions. It was found, however, that when the cells were dried previous to the respiratory experiment they were no longer able to oxidize pyruvic acid, but retained their ability to oxidize lactic acid, although the

TABLE VI

*Comparison of Effect of Calcium Pantothenate on Dried and Normal Cells*

	Lactate		Pyruvate	
	O <sub>2</sub> in 2 hrs.	Stimulation	O <sub>2</sub> in 2 hrs	Stimulation
	c. mm.	per cent	c. mm.	per cent
Intact cells ..	156		67.5	
" " + 20 $\gamma$ Ca pantothenate ..	284	82	134	100
Dried cells	126.9		0	
" " + 20 $\gamma$ Ca pantothenate	170.8	25.8	0	0

total oxygen uptake on lactic acid was reduced. Concomitant with this loss in ability to oxidize pyruvic acid, the stimulation by pantothenic acid disappeared even when lactate was used as substrate.

Table VI indicates the results of such an experiment. The cells were separated into two portions, one of which was dried and the other used without drying. The results on the intact cells showed marked stimulation when both pyruvic acid and lactic acid were used as substrates. On drying, however, it will be noted that pyruvic acid is no longer oxidized, while the stimulation on lactic acid was markedly reduced. In some experiments a small stimulation by pantothenic acid on dried cells was obtained, while in others it was completely abolished. In all cases it was reduced to such an extent as to leave no doubt as to the nature of the results. It can be concluded that pantothenic acid is not concerned in the oxidation of lactic acid to pyruvic acid, but is concerned rather with the further oxidation of pyruvic acid.

That lactic acid was being oxidized to pyruvic acid was demonstrated

directly by the isolation of pyruvic acid as the 2,4-dinitrophenylhydrazone from a reaction mixture containing *dried* cells and lactic acid. The hydrazone was identified by a mixed melting point with a known sample prepared from pure pyruvic acid (m.p. 218–222° uncorrected). That the dried cells were unable to oxidize pyruvic acid was further indicated by the following facts. It was found that when either lactic acid or pyruvic acid was used as substrate with intact cells, acetic acid could be demonstrated qualitatively in the reaction mixture. The acetic acid was determined by a modification of the lanthanum nitrate reaction as described above. When dried cells were utilized, no acetic acid could be demonstrated.

TABLE VII  
*Ratio of Pyruvate Used to Oxygen Consumed*

Unstimulated cells	Stimulated cells
2.50	2.40
3.70	2.30
4.00	2.40
2.20	2.00
2.26	1.97
2.26	1.74
2.16	1.89
1.97	1.83
1.90	1.70
2.27	2.08
2.09	1.43
2.36	2.36

The R.Q. of dried cells was found to be close to 0. This is in agreement with the above postulation that under these conditions lactic acid is converted to pyruvic acid and the pyruvic acid is not further oxidized.

The results given thus far indicate that in the metabolism of *Proteus morganii* pantothenic acid is concerned with the metabolism of pyruvic acid. In view of this conclusion it becomes of importance to determine the nature of the oxidation of pyruvic acid by these organisms.

The evidence thus far presented tends to indicate that pyruvic acid is oxidized by these organisms to acetic acid and CO<sub>2</sub>. Closer study, however, casts some doubt on the certainty of this conclusion. It will be noted in Table VII that although the pyruvate to O<sub>2</sub> ratio is close to 2.0 in most experiments, the addition of pantothenic acid (the stimulated cells) results in a lowering of this ratio. The large number of reactions which pyruvate is capable of undergoing makes the explanation of these data complex. It is possible that in the absence of added pantothenic acid some pyruvate

is metabolized through a pathway requiring less than an atom of oxygen per molecule of pyruvate and that the addition of pantothenic acid results in the preferential use of the pyruvate for oxidation to acetic acid, thus explaining the close approximation of the pyruvate to  $O_2$  ratio to 2.0 on stimulated cells. Another explanation might be that the ratio we are studying is the summation of two or more pyruvate reactions and that the addition of pantothenic acid results in a more complete oxidation (i.e., the oxidation of acetic acid), thus resulting in a lowering of the pyruvate to  $O_2$  ratio. This explanation is less likely, since the ratio never drops much below 2.0 and since the r.q. always is very close to 2.0.

In order to obtain more data on this question a study was made of the ability of these organisms to oxidize acetic acid. It was found that acetic acid was oxidized, but with a maximum oxygen uptake of only one-third that obtained with pyruvic acid. The oxygen uptake with acetic acid as substrate was stimulated by pantothenic acid, but the magnitude of the oxidation and of the stimulation was not sufficient to explain the results obtained with pyruvate.

Interpretation of the results obtained with acetate is difficult owing to the complete lack of knowledge of the manner in which acetate is utilized. It is possible that the stimulation of acetate oxidation is due to the reformation from acetate of pyruvate. It is, however, also possible that the small oxygen consumption of acetate is an artifact, since oxidation of acetate may involve acetyl phosphate. An erroneous picture might then be obtained when free acetate is added, since the limiting reaction might be the phosphorylation of acetate.

#### SUMMARY

The results presented in this paper show that pantothenic acid is concerned in certain metabolic reactions of *Proteus morganii*. The evidence indicates that pantothenic acid is concerned with the metabolism of pyruvic acid. The mechanism by which it participates in these reactions is unknown.

Although most of the evidence indicates that pantothenic acid is concerned in the conversion of pyruvic acid to acetic acid, the possibility cannot be ruled out that it acts on some other metabolic step involving pyruvic acid or some intermediate derived from pyruvic acid.

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# THE CHOLINE OXIDASE ACTIVITY OF FATTY LIVERS

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It has been found that the choline content of fatty livers produced by "labile methyl deficiency" is no less and may be greater than the choline content of normal rat livers (1). It was suggested that this may be due to an inhibition of the choline oxidase by the increased fat content (1) similar to that produced *in vitro* by fatty acids (2) and that choline cannot enter into transmethylation reactions until it has been oxidized. The following experiments show that the choline oxidase activity of fatty livers is considerably depressed.

## EXPERIMENTAL

Fatty livers in young rats were produced in 1 month on each of two diets. Diet A consisted of arachin 15, sucrose 35, rice starch 30, cottonseed oil 15, and salts (3) 5. Diet B contained casein 10, sucrose 72, cottonseed oil 10, salts (3) 7, and, in addition, nicotinamide 1. All rats received a daily supplement of 20  $\gamma$  of thiamine chloride, 20  $\gamma$  of pyridoxine, 40  $\gamma$  of riboflavin, 240  $\gamma$  of calcium pantothenate, 10  $\gamma$  of 3-methyl-1,4-naphthohydroquinone diacetate, and 2 drops daily of a mixture of wheat germ and cod liver oils. The mechanism of fatty liver formation on the 1 per cent nicotinamide diet will be described in detail in a subsequent publication.<sup>1</sup> The livers produced by this diet contained about 25 per cent lipids, while the lipid content of the livers of rats which received Diet A varied between 30 and 38 per cent.

Liver preparations were obtained by grinding the tissue in an equal volume of 0.05 M phosphate buffer, pH 7.8, and filtering through muslin. 2.0 mg. of choline or succinic acid were added and the final volume in the Warburg vessel was 2.0 ml. Measurements were made at 5 minute intervals for 15 minutes in air at 37°, pH 7.8.

Because of the difficulties inherent in the preparation of a standard broken cell suspension of liver, the choline oxidase activity was in each case compared with the succinoxidase activity of the same preparation. Many control experiments with the livers of normal rats have shown that the  $QO_2$  ratio of choline to succinate is quite constant under the conditions

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<sup>1</sup> Handler, P., unpublished data.

used, varying only between 0.55 and 0.70. Table I shows the effect of different concentrations of a typical normal rat liver suspension on this ratio. Below concentrations of 0.4 ml. of the standard 1:1 liver suspension in a total of 2.0 ml. in the Warburg vessel the ratio falls off sharply. In the present comparisons 0.4 ml. of the suspension of normal liver was used in each instance.

The fatty livers used in this study had a mean lipid content of 35 per cent. When such livers were compared with those of normal rats, it was

TABLE I

*Effect of Different Concentrations of Normal Liver Suspension on Rate of Oxidation*  
pH 7.8, 37°, 2.0 mg. of each substrate were used.

Liver suspension	$\frac{QO_2 \text{ choline}}{QO_2 \text{ succinate}}$			
	0.2 ml	0.4 ml	0.6 ml	0.8 ml
min				
5	0.51	0.75	0.84	1.00
10	0.42	0.72	0.84	0.92
15	0.41	0.70	0.82	0.92

TABLE II

*Oxidation of 2.0 Mg of Choline and 2.0 Mg of Succinic Acid by 65 Mg (Dry Weight) of Normal Rat Liver and by 158 Mg (Dry Weight) of Fatty Liver Produced by Ingestion of Diet A*

Bath temperature 37°, pH 7.8, the respective control uptakes have been subtracted

Time	Normal liver			Fatty liver		
	Choline $O_2$ uptake	Succinate $O_2$ uptake	$\frac{QO_2 \text{ choline}}{QO_2 \text{ succinate}}$	Choline $O_2$ uptake	Succinate $O_2$ uptake	$\frac{QO_2 \text{ choline}}{QO_2 \text{ succinate}}$
min	c mm	c mm		c mm	c mm	
5	44	70	0.63	7	46	0.15
10	74	129	0.57	16	94	0.17
15	96	167	0.58	26	136	0.19

necessary to compensate for the dilution effect of the fat. When livers from rats on Diet A were employed, the ratio of dry weight of fatty liver to dry weight of normal liver, in their respective Warburg vessels, was 2.5:1. For livers of animals which had received Diet B the ratio employed was 2:1. This was more than ample compensation for the diluting effect of the fat and should have tended to increase the  $QO_2$  ratio of choline to succinate for these livers as shown in Table I.

Table II is the protocol of a typical comparison between a fatty liver

produced by the ingestion of Diet A and a normal liver. While there was apparently a decrease of 20 per cent in the succinoxidase activity of the fatty liver, there was a decrease of 80 per cent in the choline oxidase activity (without considering the discrepancy in the dry weights of tissue used).

This was further reflected by the 65 per cent drop in the  $QO_2$  ratio of choline to succinate. When stearic acid was added to suspensions of normal rat livers, the succinoxidase activity was inhibited 19 per cent while the choline oxidase activity decreased 61 per cent (2). The results with added fatty acid and with fatty livers are, thus, in good agreement. It must be remembered, however, that the molecular concentration of neutral fat in these studies was more than 30 times as great as that of the stearic acid in the former experiments.

When fatty livers produced by feeding Diet B were employed the results were not quite as striking. Again there was a small drop in succinoxidase activity. However, the choline oxidase activity was but 50 per cent less than that found in the normal controls. The mean values of  $QO_2$  ratio of choline to succinate for such livers at 5, 10, and 15 minutes were 0.31, 0.38, and 0.35 respectively.

It was originally reported that fatty acids inhibited the choline oxidase but that neutral fat did not. It is possible that added neutral fat did not become intimately mixed with the tissue suspension and that this was the reason for the negative results obtained. In the fatty liver the fat is originally incorporated within the cell and is thus presumably in contact with the tissue proteins and enzymes. While the inhibition of the choline oxidase in these fatty livers was not complete, these data do appear to support the hypothesis of Jacobi and Baumann.

#### SUMMARY

1. In normal rat livers, the ratio of the rate of oxidation of choline to that of succinic acid was found to be 0.60 under the conditions described.
2. In fatty livers produced on a low methionine diet this ratio was found to decrease to 0.20.

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per day. We have cured twelve rats of this paralysis or spasticity over a 2 to 3 week period of therapy. After these results were confirmed, it seemed desirable to investigate the effect of crystalline biotin. We had a group of four rats which showed severe paralysis. Two rats were given the methyl ester of biotin at a level of 1  $\gamma$  per day for 10 days and the other two rats were given biotin (acid) at the same level for 10 days. The four rats were then observed over a period of 3 to 4 weeks. In all the animals, a complete cure was noted at the end of this period. In order to give some idea of the responses observed, Fig. 1 was taken at the end of the 14th week on a high fat ration (weight 138 gm.); Fig. 2 portrays the same rat after it had been given 1  $\gamma$  of biotin for 10 days and then allowed an additional 16 days to recover (weight 190 gm.). The response to the free



FIG. 1

Fig. 1. A biotin-deficient rat, body weight 138 gm.



FIG. 2

Fig. 2. The same rat as in Fig. 1, given 1  $\gamma$  of biotin per day for 10 days and then allowed an additional 16 days to recover; body weight 190 gm.

biotin seemed to be more rapid than that observed with the methyl ester of biotin. Since we did not feed limiting levels and since the methyl ester contains about 10 per cent of its total activity as free biotin, it is difficult to draw a definite conclusion, but it would appear that the rat does not utilize the ester as efficiently as the free biotin.

It was possible to maintain rats for 25 weeks on the ration described if at the 14th week 1  $\gamma$  of biotin per day was administered for a 2 week period. At autopsy such animals at the 25th week showed no pathological changes other than those characteristic of a mild biotin deficiency. It would thus appear that biotin was the only limiting factor in this diet.

A fat-free ration was devised by replacing the corn oil in our ration with sucrose. Four rats were placed on this ration and the typical paralysis was noted in all the animals at the end of 9 weeks. Throughout these

studies we have had thirty rats on the ration described and the onset of this symptom appears in from 9 to 12 weeks. The level of fat in the ration was studied, since high levels of fat in a riboflavin-low ration increased this type of paralysis (6). The high fat ration employed had the following percentage composition: sucrose 28, casein 11, commercial egg white 14, Salts 4 (7) 6, and lard 41. Choline hydrochloride was added at 3 gm. per kilo of ration. This ration contains 41 per cent of fat and the sucrose was replaced isocalorically by lard. Six rats were maintained on this ration while the riboflavin supplementation was increased to 100  $\gamma$  per day. The

TABLE I  
*Creatine Content of Muscle*

Animal No.	Period on ration	Final weight	Degree of paralysis	Creatine
	<i>wks.</i>	<i>gm.</i>		<i>mg. per gm.</i>
1	13	90	Spastic	4.48
2	13	188	"	4.36
3	13	120	"	4.61
4	13	120	Normal*	3.23
5	14	195	Slight, spastic	3.85
6	9	68	Spastic	4.30
7	9	170	Normal†	3.62
8	9	173	" ‡	3.90
9	14	114	Spastic	4.53
10	15	140	" §	3.46
11	15	191	Normal	3.66
12	15	140	Slight, spastic¶	4.05
13	15	120	Spastic	4.52
14	9	72	"	4.44

\* Basal ration; cured with biotin over a 3 week period.

† Basal ration with 1  $\gamma$  of biotin given orally as a daily supplement.

‡ Basal ration with egg white replaced by casein.

§ High fat ration.

|| Fat-free ration; cured with biotin.

¶ Fat-free ration; partially cured with biotin.

onset of spasticity was materially lengthened, usually occurring in from 12 to 14 weeks. Three additional animals were maintained on the high fat ration but received a normal level of riboflavin. The onset of spasticity was 12 to 14 weeks. From these studies it appears that fat has a slight sparing action, in contrast to the results obtained in rats fed a riboflavin-low ration. Albino rats develop a biotin deficiency sooner than piebald rats, but piebald rats were used in all the studies reported in this paper.

The effect of riboflavin and pyridoxine was studied in the following manner. A group of four rats was placed on the basal ration with the



riboflavin supplementation increased to 100  $\gamma$  per day. All the animals developed the paralysis in from 10 to 12 weeks. Another group of four animals was given 100  $\gamma$  of riboflavin per day by intraperitoneal injection; again the onset of paralysis was not changed. Four additional rats were maintained on the basal ration, the riboflavin was increased to 120  $\gamma$  (orally) per day, and pyridoxine was increased to 75  $\gamma$  per day with no noticeable effect. In another group of four rats the pyridoxine supplementation was increased to 100  $\gamma$  per day and the paralysis developed over a period of 8 to 11 weeks. Judging from the results of these sixteen animals it appears that riboflavin, pyridoxine, or the combination of the two vitamins had no effect on this paralysis.

Muscle creatine was studied in the following manner. The animals were killed by stunning and samples of muscle taken from the thigh of the hind leg. Creatine (total creatine) was determined by the method of Rose, Helmer, and Chanutin (8), with the following modification. A 1 ml. aliquot of the clear filtrate was diluted with distilled water to 10 ml. in an Evelyn tube, the alkaline picrate added, and the color read in the colorimeter with a No. 520 filter. This method is not specific for creatine but Baker and Miller (9) have shown that the error is probably less than 10 per cent in the analysis of skeletal muscle. The creatine values are given in Table I. The rats which were biotin-deficient gave higher creatine values. The animals which were partially cured by means of biotin administration gave values which were approaching the normal values obtained. The musculature of the biotin-deficient animals had a sinewy appearance, but did not have a lower water content.

#### DISCUSSION

A typical paralysis or spasticity of the hind legs develops in biotin-deficient rats. This paralysis has been produced very consistently by the inclusion of egg white in a synthetic diet which allows good growth in the absence of egg white. A similar paralysis has been produced in rats maintained on a riboflavin-low ration by Mannering, Lipton, and Elvehjem (6). Under their conditions high levels of fat in the diet exaggerated the paralysis. High levels of fat in our ration did not hasten the onset of this spasticity but had a slight protective action. The paralysis reported in this paper was not corrected by riboflavin. It appears that riboflavin is necessary for the optimum synthesis of biotin in the intestinal tract of the rat (unpublished data). Since riboflavin appears to be involved in the synthesis of biotin, the riboflavin-low ration may be a means of producing an imbalance or a mild biotin deficiency. Biotin-deficient rats are very sensitive to handling in the lumbar area of the spine and this is also characteristic of the riboflavin-deficient animals.

The muscle creatine of biotin-deficient rats is abnormally high. The administration of biotin decreases the muscle creatine. The paralysis observed is not due to low creatine levels, an abnormality found in muscle dystrophy of vitamin E-low rats investigated by Knowlton and Hines (10) and Telford, Emerson, and Evans (11).

Findlay and Stern (3) in studies with rats maintained on an egg white ration have reported a lesion in the spinal cord and slight but definite changes in the peripheral nerves. These investigators used a ration which was low in riboflavin and may have observed the lesions found by Shaw and Phillips (12) as being characteristic of a riboflavin deficiency. Our animals did not show any gross pathology of the nerves as is the case with riboflavin-deficient rats. The tissues of the biotin-deficient animals have been given to Dr. P. H. Phillips and the histological studies will be reported in a separate paper.

#### SUMMARY

1. A paralysis is produced in rats by the inclusion of low levels of egg white in a synthetic ration. The administration of biotin concentrates or biotin is specific for this syndrome.

2. High levels of fat in the diet slightly prolong the onset of paralysis.

3. Riboflavin, pyridoxine, or the combination of the two vitamins was without effect.

4. High creatine levels were observed in the leg muscle of paralytic rats.

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# A COMPARISON OF THE ALKALINE CLEAVAGE PRODUCTS OF TWO STRAINS OF TOBACCO MOSAIC VIRUS

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Two fundamentally different possibilities for the structure of high molecular weight nucleoproteins of the virus type were recently suggested by Pfankuch (1). One alternative was represented by an enormous protein component to which nucleic acid side chains were attached, while the other possibility involved a chain-like structure consisting alternately of small protein and nucleic acid units.

Pfankuch removed nucleic acid from tobacco mosaic virus and from a variant (TM44) by means of either alkali or pyridine and studied the proteins thus obtained in the Tiselius electrophoresis apparatus (1). Each protein appeared to be electrochemically homogeneous, and, although a mixture of the intact viruses had given a double boundary, a mixture of the nucleic acid-free proteins yielded a single boundary. From this observation and from one solubility test, it was concluded that the proteins obtained from the two strains were chemically identical while the nucleic acids were different. Since the protein derived from each strain exhibited no specific turbidity in a specially prepared neutral solution, Pfankuch concluded that it was of low molecular weight. From these experiments, Pfankuch was led to draw the general conclusions that the structure of virus nucleoproteins was best represented by the chain hypothesis in which small protein units are linked alternately with nucleic acid units, and that the differences between tobacco mosaic virus and its variant strains can be attributed to quantitative or qualitative differences in the nucleic acid component of the virus molecule. The latter conclusion had been suggested earlier by Pfankuch, Kausche, and Stubbe when they observed a 15 per cent difference between the phosphorus contents of two strains of tobacco mosaic virus (2).

Evidence was recently presented from this laboratory which, contrary to Pfankuch's conclusions, showed that, in the case of spontaneously arising strains of tobacco mosaic virus, the chemical differences between strains probably lie not in the nucleic acid but rather in the protein part of the virus molecule (3). For example, tyrosine, tryptophane, and phenylalanine contents of 6.4, 3.5, and 4.3 per cent, respectively, were found for the Holmes rib-grass strain as compared to the strikingly different values of

3.8, 4.5, and 6.0 per cent, respectively, established for ordinary tobacco mosaic virus. Moreover, the rib-grass virus was found to contain about 3 times as much sulfur as ordinary tobacco mosaic virus<sup>1</sup> and to contain histidine, an amino acid which appears to be entirely lacking in tobacco mosaic virus.<sup>2</sup> Qualitative tests showed that both viruses contained the yeast type of nucleic acid. Phosphorus analyses gave values of 0.54 and 0.56 per cent, a fact which may be considered an indication of the absence of significant quantitative differences in the nucleic acid component of the viruses.

In view of the definite differences in the amino acid contents of rib-grass and ordinary tobacco mosaic viruses, it seemed worth while to investigate these viruses and their protein components by the electrophoretic technique, in order to test the sensitivity of this method to small but significant differences in the composition of related proteins. It was believed that this study might afford a critical test of Pfankuch's assumption that electrochemical homogeneity of the mixed alkaline cleavage products of two viruses indicated that the proteins of those viruses were chemically identical. Moreover, it was hoped that such an investigation would yield information regarding the structure of nucleoproteins of the tobacco mosaic virus group.

#### EXPERIMENTAL

*Preparation of Materials*—The general properties of tobacco mosaic virus are well known. The general characteristics and some of the physical and chemical properties of the rib-grass strain have recently been described<sup>1</sup> (4). In the present experiments, each virus was obtained from appropriately diseased Turkish tobacco plants. The viruses were isolated from the infectious juices of these plants and were highly purified by the customary cycles of high and low speed centrifugation.

Nucleic acid-free protein<sup>3</sup> was prepared from each virus by adding dropwise and with stirring enough 50 per cent sodium hydroxide to 50 ml. of an ice-cold 0.2 per cent solution of virus in distilled water to bring the total concentration of alkali to 5 per cent. After 2 minutes, the solution was neutralized to litmus with glacial acetic acid. 15 minutes later the precipitated protein was separated by centrifugation and washed two times with 15 to 20 ml. of cold distilled water. The protein was next dissolved in 10 ml. of 0.01 N sodium hydroxide and reprecipitated by the addition of an equal volume of saturated sodium sulfate. The precipitate was isolated by

<sup>1</sup> Knight, C. A., *J. Biol. Chem.*, in press.

<sup>2</sup> Knight, C. A., unpublished data.

<sup>3</sup> The nucleic acid-free materials will be referred to throughout this report as virus proteins, and the intact viruses will be referred to as such or will be called by their respective names; i.e., tobacco mosaic virus and rib-grass virus.

centrifugation, washed once with 15 ml. of cold water, and finally dissolved in 10 ml. of 0.01 *N* sodium hydroxide. The alkaline solution of protein was centrifuged for 10 minutes at 30,000 R.P.M. and the small amount of residue obtained in this step was discarded. Yields of about 75 per cent of the theoretical were generally obtained.

Phosphorus and carbohydrate analyses demonstrated that essentially all of the nucleic acid had been separated from the protein component of each virus. The absence of nucleic acid from the protein preparations was also shown by the results of ultraviolet absorption studies carried out by Dr. G. I. Lavin and described in detail elsewhere.<sup>1</sup> The intact viruses showed a maximum absorption at about 2675 Å., and the proteins showed a maximum absorption at about 2800 Å. The relative positions of maxima and minima and the shapes of the curves were those to be expected from nucleoproteins and simple proteins, respectively. In addition, further evidence of the absence of intact virus from the preparations was obtained when activity tests of the proteins on *Nicotiana glutinosa* L. yielded no lesions.

*Electrophoresis Experiments*—Electrophoresis experiments were performed in the Tiselius apparatus with the Longworth schlieren scanning method (5). All materials were dialyzed to equilibrium against the buffer to be used in the electrophoresis cell.

The intact viruses were examined first separately and then in a mixture. These experiments were performed in 0.05 *M* phosphate buffer at pH 7.1 with concentrations of protein from 2 to 4 mg. per ml. A single sharp boundary was obtained for each virus, while two sharp boundaries were obtained for the mixture of viruses. The mobilities of tobacco mosaic virus and the rib-grass strain calculated from the descending boundaries in the individual runs were  $-8.8 \times 10^{-5}$  and  $-7.6 \times 10^{-5}$  cm. per second per volt per cm., respectively. The mobilities of the two viruses in the mixture were  $-8.9$  and  $-7.8 \times 10^{-5}$ .

The nucleic acid-free proteins obtained from the two viruses by the procedure described above were next mixed in equal quantities and examined. Experiments were performed in 0.05 *M* phosphate buffer at pH 7.1 and also in 0.1 *N* sodium hydroxide-0.2 *N* glycine buffer at pH 10. In some cases the virus proteins were mixed before and in other cases after dialysis. Single boundaries were obtained in the electrophoresis apparatus in all cases. These experiments demonstrated that mixtures of two chemically different virus degradation products may show only a single boundary in the electrophoresis apparatus. Hence, failure to resolve a mixture of proteins by this method cannot be regarded as even reasonable evidence of the chemical identity of the components, as was assumed by Pfankuch. A more detailed consideration of the results of the present study, however, revealed

that the single boundaries obtained with mixtures of virus proteins may undergo some reversible spreading. This could be due to a slight difference between the mobilities of the two components of the mixture. Such slight resolution as this, however, could not be detected by the schlieren band method used by Pfankuch.

*Amino Acid Analyses*—The single boundary obtained in the electrophoretic study of the mixed alkaline cleavage proteins made it seem desirable to demonstrate that the differences in amino acid composition known to exist in the intact viruses were still present in the nucleic acid-free materials. This was accomplished by analysis of each material for aromatic amino acids.

A portion of each cleavage protein was dialyzed against flowing distilled water until precipitation occurred. The protein was then sedimented in an angle centrifuge, washed twice with cold water, frozen, and dried *in vacuo*, and finally further dried at 110° in an oven. The white powder thus obtained was hydrolyzed and analyzed as described recently for the intact viruses (3). Tyrosine, tryptophane, and phenylalanine values of 6.7, 3.4, and 4.65 per cent, respectively, were found for the rib-grass virus protein compared to 4.05, 4.65, and 6.5 per cent, respectively, for the ordinary tobacco mosaic virus protein. These values indicated that the alkaline cleavage products had retained chemical differences noted in the intact viruses, and that the single boundaries obtained with mixtures of the alkaline cleavage proteins in the electrophoresis experiments were actually obtained with two materials of different chemical compositions.

*Size of Cleavage Products*—One preparation of each nucleic acid-free protein was examined in a rocking osmometer at 6°. Owing to the limited solubility of the proteins, it was necessary to perform the experiments in a less concentrated salt solution than might otherwise have been desirable. The sodium hydroxide-glycine buffer which was used in one of the electrophoresis experiments was employed. About 1 per cent solutions of the proteins developed negligible pressures in 5 days. This result suggested that the particle weight of the material under the above conditions was at least several hundred thousand.

Attempts were next made to ascertain the size of the protein by means of the analytical ultracentrifuge. A mixture composed of 2 mg. of each of the intact viruses gave a single boundary with  $S_{20} = 188 \times 10^{-13}$ . This value is in reasonable agreement with that obtained for tobacco mosaic virus alone (6). However, difficulty was encountered with the nucleic acid-free proteins, for it quickly became apparent that both proteins possessed a tendency to aggregate to form large particles which sedimented almost immediately. This phenomenon was particularly pronounced with both proteins in phosphate buffer at pH 7.1 but also occurred with tobacco mo-

saic virus protein in sodium hydroxide-glycine buffer at pH 10. Moreover, rise in temperature appeared to accelerate aggregation in almost every case. However, the rib-grass virus protein remained comparatively unaggregated in the buffer at pH 10 and gave a single boundary in the ultracentrifuge. Three different preparations of rib-grass virus protein containing 5 mg. of protein per ml. gave sedimentation constants of 25, 27, and  $30 \times 10^{-13}$ . If it is assumed that these products are spheres with a partial specific volume of about 0.735, the average of these values would correspond to a molecular weight of about  $7 \times 10^5$ . It was next found possible to obtain a boundary in the ultracentrifuge with tobacco mosaic virus protein if the protein were dissolved in 0.01 M sodium hydroxide. With 1 per cent solutions of the protein, sedimentation constants of 11 and  $15 \times 10^{-13}$ , corresponding to molecular weights of  $1.2 \times 10^5$  and  $3 \times 10^5$ , were obtained for two different preparations. In order to obtain comparative values, the rib-grass virus protein was also centrifuged in 0.01 M sodium hydroxide. Sedimentation constants of 4.6 and  $5.9 \times 10^{-13}$ , corresponding to molecular weights of 5 and  $7 \times 10^4$ , were obtained with 0.01 M alkali solutions containing 11.9 and 10 mg. of protein per ml., respectively. When a 1 per cent solution in 0.01 M sodium hydroxide of the tobacco mosaic virus protein with a sedimentation constant of  $11 \times 10^{-13}$  was mixed with a similar 1 per cent solution of the rib-grass virus protein with a sedimentation constant of  $4.6 \times 10^{-13}$ , a single boundary was obtained in the ultracentrifuge with a sedimentation constant of  $16 \times 10^{-13}$ , from which a molecular weight of about  $3.3 \times 10^5$  could be calculated. This somewhat surprising result appeared to indicate the formation of a mixed aggregate of the two proteins.

Solutions of the proteins in 0.01 M sodium hydroxide were also used in a study with the electron microscope. As described in a recent communication, the intact viruses are rod-shaped, and there appears to be no striking difference in the average size of their particles.<sup>1</sup> Electron micrographs of the alkaline cleavage proteins demonstrated particles which reflected the previously noted tendency of the materials to form aggregates. The tobacco mosaic virus protein appeared quite heterogeneous in size and shape, although none of the material approached the size and shape of the intact virus. Assuming a spherical shape for the smaller particles in the micrographs, it was possible to calculate a molecular weight which agreed roughly with that calculated from the sedimentation constant for the same material. Micrographs of the rib-grass virus protein either showed no particles or occasionally a few masses of material which presumably might have been formed during preparation of the mounts. This was to be expected from the sedimentation data, for the apparent size of the material in 0.01 M alkali was such as to be practically invisible in the electron microscope. Micrographs of a mixture of tobacco mosaic and rib-grass virus proteins



showed an appreciable amount of material, but again there was no uniformity in the size and shape of the particles. The electron micrographs referred to above were kindly made by Dr. T. F. Anderson, RCA Fellow of the National Research Council.

#### DISCUSSION

The various strains of tobacco mosaic virus are closely related nucleoproteins; nevertheless, these virus strains differ in subtle biological specificities. According to our present knowledge, such differences in biological properties are probably manifestations of differences in the structure or in the composition either of the nucleic acid or of the protein constituents of the various virus molecules. Pfankuch considered that the differences reside in the nucleic acid portions, and he based his conclusion on the observation that a mixture of the protein components of two strains gave a single boundary in an electrophoresis experiment. In the present investigation, a mixture of two virus proteins with unequivocal chemical differences was found to show a single boundary in the electrophoresis apparatus. Therefore, Pfankuch's conclusions from similar data are left without material support. The latter fact does not, however, exclude the possibility that strains of tobacco mosaic virus may differ with respect to their nucleic acid components, although at present there is little direct chemical evidence to that effect.

The authors wish to express their appreciation to Dr. W. M. Stanley for help and encouragement during the course of this investigation.

#### SUMMARY

Nucleic acid was removed by means of alkali from ordinary tobacco mosaic virus and a rib-grass strain of tobacco mosaic virus. The nucleic acid-free protein components of the viruses were examined in the Tiselius electrophoresis apparatus, in the analytical ultracentrifuge, and in the electron microscope. Although it was shown that the protein components differed in their amino acid composition, a mixture of the proteins at pH 7.1 and at pH 10 migrated in an electrical field as though composed of a single component. From these experiments, it was concluded that the electrophoretic technique could not be depended upon to distinguish between biologically related but chemically different proteins. It was found possible under certain conditions to obtain sedimentation constants and, hence, estimates of the average size of the protein particles in several preparations. The smallest and largest materials represented by different preparations corresponded to molecular weights of  $5 \times 10^4$  and  $7 \times 10^5$ . Electron micrographs of the proteins showed particles of many sizes, some of which corresponded to those calculated from sedimentation data.

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# RELATION OF THE CONCENTRATION OF STARCH SUSPENSIONS TO THEIR VISCOSITY\*

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Twelve of the observations in Table I were made by pipetting 10 cc. of 0.1 M phosphate mixture at pH 5.6 (95 cc. of 0.1 M  $\text{KH}_2\text{PO}_4$  + 5 cc. of 0.1 M  $\text{Na}_2\text{HPO}_4$ ) into an Ostwald viscosimeter and determining its viscosity (the outflow time of 5 cc.) at 34°, and then successively withdrawing 1 cc. of the phosphate mixture and replacing it by 1 cc. of a fresh 5 per cent suspension of Lintner soluble starch<sup>1</sup> and determining the viscosity of the resulting suspensions. This procedure was repeated twelve times. The first solution was starch-free, the second contained 50 mg. of starch, the third contained 95 mg. of starch (50 - 5 + 50), etc., increasing the starch concentration until the twelfth suspension containing 358.79 mg. of starch in 10 cc. was reached.

The remaining eight observations recorded in Table I were made by pipetting 10 cc. of the fresh 5 per cent starch suspension into an Ostwald viscosimeter and determining its viscosity at 34°, and then successively withdrawing 1 cc. of this starch suspension and replacing it by 1 cc. of 0.1 M phosphate mixture, and determining the viscosity of the resulting suspensions. This procedure was repeated eight times. The first suspension contained 500 mg. of starch in 10 cc. of 0.1 M phosphate mixture at pH 5.6, the second contained 450 mg. of starch (500 - 50), the third contained 405 mg. of starch (450 - 45), etc., decreasing the starch concentration until the eighth suspension containing 239.15 mg. of starch in 10 cc. was reached.

A plot of the relative viscosities observed in these two series of measurements against the respective starch concentrations (gm. per 100 cc.) indicated an agreement to within about  $\pm 2.5$  per cent, which may be taken as the basic error of the method employed.

\* This work was carried out with the greatly appreciated assistance of Dr. Hans Neurath of the Department of Biochemistry, Duke University School of Medicine.

<sup>1</sup> All of the studies in Table I were made with the same preparation of soluble starch (Merek, made according to Lintner). Those in Table II were made with another preparation of Lintner soluble starch. The viscosity of starch suspensions varies with the particular lot of soluble starch employed. This variation bears but little relation to the water content of the starch. The sample of Lintner starch used in Table I had 10.1 per cent moisture, and that in Table II had 9.2 per cent.

Inspection of the data indicated a non-linear relation between relative viscosity and solute concentration, as has been observed with most solutions of high polymeric substances (1). The data did not satisfy the logarithmic relation of Arrhenius (2), but appeared to follow, within the limits of the experimental error, the concentration-fluidity relation of Treffers (3)

TABLE I  
*Relation of Concentration of Starch Suspensions (Made by Dilution) to Their Relative Viscosity and Specific Fluidity*

Concentration of starch suspension, $c$ (1)	Outflow time of 5 cc. starch suspension, $\eta^*$ (2)	Relative viscosity, $\frac{\eta}{\eta_0}$ (3)	Specific fluidity, $\frac{\eta_0}{\eta} - 1$ (4)	Concentration-fluidity factor, $\frac{\eta_0/\eta - 1}{c} = k$ (5)
<i>per cent</i>	<i>sec.</i>			
5.00 (Decreasing)	104.3	2.125	-0.529	0.106
4.50 "	96.3	1.96	-0.490	0.109
4.05 "	89.9	1.83	-0.454	0.112
3.65 "	84.3	1.71	-0.415	0.113
3.59 (Increasing)	80.0	1.63	-0.387	0.107
3.43 "	78.7	1.60	-0.375	0.109
3.28 (Decreasing)	79.5	1.62	-0.383	0.117
3.26 (Increasing)	76.6	1.56	-0.360	0.111
3.06 "	74.6	1.52	-0.342	0.109
2.95 (Decreasing)	75.4	1.53	-0.347	0.117
2.85 (Increasing)	72.0	1.47	-0.318	0.111
2.66 (Decreasing)	72.4	1.47	-0.322	0.121
2.61 (Increasing)	70.2	1.43	-0.301	0.115
2.39 (Decreasing)	69.1	1.41	-0.290	0.121
2.34 (Increasing)	67.8	1.38	-0.276	0.118
2.05 "	64.7	1.32	-0.240	0.117
1.72 "	61.4	1.25	-0.200	0.117
1.36 "	58.9	1.20	-0.163	0.120
0.95 "	55.2	1.12	-0.106	0.112
0.50 "	52.2	1.06	-0.057	0.114
Mean.....				0.114

\* The outflow time of 5 cc. of the buffer solution ( $\eta_0$ ) was 49.1 seconds.

$$\frac{\eta_0/\eta - 1}{c} = k$$

in which  $\eta_0/\eta$  was the reciprocal of the relative viscosity,  $c$  the solute concentration in weight per cent, and  $k$  a constant. As shown in Columns 4 and 5 of Table I, this relation was satisfied fairly closely by the present data. A plot of the specific fluidity against the starch concentration was a straight

line. The data did not admit of a further evaluation in terms of molecular volume (4) or shape (5).

However, starch suspensions made individually by weight and not by successive dilutions of a stock suspension, as in Table I, did not follow the concentration-fluidity relation of Treffers (3). For example, 1.04, 2.5, 5.0, and 7.5 gm. of Lintner soluble starch<sup>1</sup> were suspended in 100 cc. of 0.1 M phosphate solution at pH 7.0 (40 cc. of 0.1 M  $\text{KH}_2\text{PO}_4$  + 60 cc. of 0.1 M  $\text{Na}_2\text{HPO}_4$ ), boiled 1 to 2 minutes, autoclaved 15 minutes at 15 pounds pressure, one or more thymol crystals added, and the suspensions filtered, while hot, through one thickness of muslin. As shown in Table II, the concentration-fluidity factor of these four suspensions varied from 0.121 to 0.252,

TABLE II  
*Relation of Concentration of Starch Suspensions (Made by Weight) to Their Relative Viscosity and Specific Fluidity*

Concentration of starch suspension, <i>c</i>	Outflow time of 5 cc starch suspension, $\eta$	Outflow time of 5 cc buffer solution, $\eta_0$	Relative viscosity, $\frac{\eta}{\eta_0}$	Specific fluidity, $\frac{\eta_0}{\eta} - 1$	Concentration-fluidity factor* $\frac{\eta_0/\eta - 1}{c} = k$
<i>per cent</i>	<i>sec.</i>	<i>sec.</i>			
7.5	385.2	33.0	11.67	-0.91	0.121
7.5	351.4	29.5	11.91	-0.91	0.121
5.0	257.7	49.7	5.18	-0.81	0.162
5.0	248.0	48.0	5.17	-0.81	0.162
2.5	90.5	41.3	2.19	-0.54	0.216
2.5	127.4	55.8	2.28	-0.56	0.224
1.04	39.2	28.5	1.38	-0.27	0.252
1.04	40.6	29.5	1.37	-0.27	0.252

\* See reference (3).

while that of the suspensions made by dilution (Table I) varied only from 0.106 to 0.121 with a mean of 0.114. A plot of the specific fluidity against the starch concentration indicated a non-linear relation.

The probable explanation for this discrepancy is that the starch in suspensions made with Lintner soluble starch apparently exists in at least two forms: (a) microscopically visible starch granules, and (b) ultramicroscopic colloidal particles. The former were counted in a blood counting chamber and numbered from 2800 to 3950 per c.mm. in a 5 per cent suspension. After the suspension had been allowed to stand several hours, these granules sank to the bottom of the tube, but the supernatant fluid which contained the colloidal particles remained opalescent indefinitely. The viscosity of the suspension remained constant; so the colloidal particles were responsible for its viscosity. Consequently the viscosities of dilutions of a stock suspension were more uniform than those of suspensions made by weight.

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# POLYSACCHARIDE SYNTHESIS FROM GLUCOSE BY MEANS OF PURIFIED ENZYMES\*

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Although the formation of glycogen and of starch from monosaccharides in animal and plant cells has been the subject of exhaustive study since the time of Claude Bernard, an insight into the mechanism of the process has been gained only recently. The fundamental discovery by Robison (1) that a mixture of glucose- and fructose-6-phosphates is formed from hexoses in yeast extract, and the isolation by Embden and Zimmermann (2) of a similar mixture of esters from skeletal muscle, provided the basis for later studies, with intact tissues as well as with cell-free enzyme systems, in which it has been established that hexose monophosphate is a necessary intermediate in the formation of polysaccharide from hexoses.

Cori and Cori (3) showed that upon tetanic stimulation of mammalian muscle the glycogen which disappeared was considerably in excess of the lactic acid formed and that the excess was accounted for by an increase in the level of hexose monophosphate. During aerobic recovery of stimulated muscle, glycogen was resynthesized at the expense of hexose monophosphate (4). In resting frog muscle kept anaerobically in Ringer's solution, the rate of breakdown of glycogen was markedly increased by the addition of epinephrine, and hexose monophosphate accumulated in amounts equivalent to the inorganic phosphate which disappeared (5). The same effect of epinephrine was observed when iodoacetate was added to prevent esterification of inorganic phosphate with creatine, and it was concluded that hexose monophosphate was formed by direct esterification of inorganic phosphate with carbohydrate (6).

Parnas and Baranowski (7) showed that this formation of hexose-6-phosphate from glycogen and inorganic phosphate (which they termed "phosphorolysis") also took place in muscle extracts. It was then found by Cori and Cori (8) in experiments with minced frog muscle and with muscle extracts that the primary product of the reaction between glycogen and inorganic phosphate was glucose-1-phosphate; this then underwent

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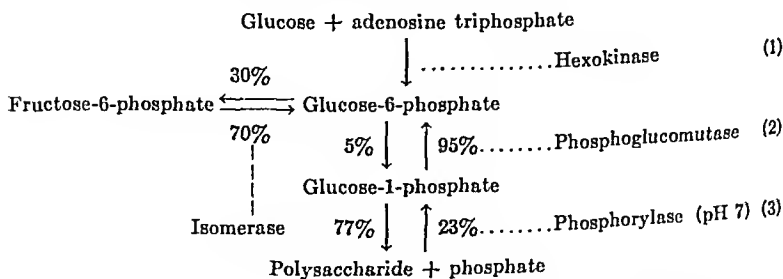


what was believed to be an irreversible intramolecular rearrangement to form glucose-6-phosphate (9). The enzymes, phosphorylase and phosphoglucomutase, which catalyze the phosphorolysis and the rearrangement, respectively, were shown to be present in a number of mammalian tissues and in yeast (10).

The reaction catalyzed by phosphorylase is now known to be reversible; *i.e.*, the formation of polysaccharide and inorganic phosphate from glucose-1-phosphate has been shown by Schäffner and Specht (11) and by Kiessling (12) with yeast phosphorylase, by Cori, Schmidt, and Cori (13, 14) with phosphorylase from muscle, liver, heart, and brain, and by several other investigators working with both plant and animal tissues (15, 16). Phosphorylase has recently been isolated in crystalline form from muscle by Green, Cori, and Cori (17).

Since glucose-1-phosphate can be converted enzymatically into polysaccharide, and since there is present in yeast and in animal tissues an enzyme, hexokinase, which catalyzes the reaction between glucose and adenosine triphosphate to form glucose-6-phosphate, it only remained necessary to show that glucose-6-phosphate could be converted to glucose-1-phosphate, in order to establish the mechanism of polysaccharide formation from glucose.

In a recent note (18) it was reported that the reaction catalyzed by phosphoglucomutase was in fact reversible and that, at equilibrium, proximately 5 per cent of the glucose monophosphate was present as 1-ester and 95 per cent as 6-ester. In spite of the unfavorable position of the equilibrium, a formation of polysaccharide from glucose-6-phosphate could be demonstrated by the combined action of phosphoglucomutase and phosphorylase, by keeping the level of inorganic phosphate low enough to allow Reaction 3 to proceed to the side of polysaccharide formation.



In this paper, a more detailed study of the reaction catalyzed by phosphoglucomutase is reported and the synthesis of polysaccharide from glucose by the successive action of hexokinase, phosphoglucomutase, and phosphorylase is demonstrated.

### Methods

The enzymes were allowed to stand in the presence of 0.5 per cent glutathione at pH 7 for 15 minutes at 30° before addition of the substrates, in order to insure optimal activity. After incubation with the substrate, the reaction was interrupted by addition of trichloroacetic acid or sodium hydroxide.

Aliquots of the trichloroacetic acid filtrates were analyzed for inorganic phosphate (a) directly, (b) after hydrolysis for 5 minutes in 0.1 N H<sub>2</sub>SO<sub>4</sub> at 100°, and (c) after ashing. Since glucose-1-phosphate is completely hydrolyzed by the short heating in acid, while glucose-6-phosphate and fructose-6-phosphate are not measurably hydrolyzed under these conditions, the amounts of 1-ester and 6-ester present may be determined readily by this procedure. Aliquots were also analyzed for fructose by the method of Roe (19), in order to determine the amount of fructose-6-phosphate present. The values were corrected, since fructose-6-phosphate gives only 48 per cent of the color given by an equivalent amount of free fructose in the Roe method. When adenosine triphosphate was to be measured, its two labile phosphate groups were split off by hydrolysis for 10 minutes in N H<sub>2</sub>SO<sub>4</sub> at 100° and determined as inorganic phosphate.

For the determination of polysaccharide, the reaction was interrupted by addition of an equal volume of 30 per cent NaOH. After digestion at 100° for 30 minutes, water was added to make the concentration of NaOH 10 per cent, 1.3 volumes of 95 per cent alcohol were added, and the mixture was boiled for a few seconds. The polysaccharide was centrifuged down and hydrolyzed with 1 cc. of N HCl for 3 hours at 100°. The hydrolysate was analyzed for glucose by the method of Shaffer and Somogyi (20).

### Materials

#### *Preparation of Enzymes*

The chief problem which arose in this work was that of obtaining the desired enzymes free from interfering enzymes which catalyzed side reactions. In this section, the interfering enzymes encountered in each type of enzyme preparation and the methods finally adopted for their removal will be described.

*Muscle Extract*—Aqueous extracts of rabbit muscle were allowed to stand for 2 hours at 25° (in order to reduce the content of adenylic acid by deaminase action), and dialyzed in collodion bags for 15 hours against running tap water at 10°. In some cases the dialyzed extracts were frozen in a thin layer and concentrated (or dried) *in vacuo* by using a trap immersed in a dry ice-ethanol mixture.

Although such extracts are able to convert glucose-6-phosphate to poly-

saccharide, the increase in polysaccharide determined by analysis may be only a fraction of that calculated from the amount of inorganic phosphate formed. Aqueous extracts of rabbit skeletal muscle do not contain phosphatase. The discrepancy is due to the presence of diastase. Part of this enzyme may be intracellular, since prolonged perfusion of muscle prior to extraction does not completely remove it.

*Phosphorylase*—Muscle phosphorylase preparations which have undergone repeated fractionation with ammonium sulfate retain some diastase activity, even when possessing a phosphorylase activity as high as 50 per cent of that of the crystalline enzyme. It was therefore necessary to use the crystalline phosphorylase in order to obtain complete analytical agreement between polysaccharide and inorganic phosphate formation. A preparation of pure phosphorylase which had been crystallized five times was kindly supplied by Dr. A. A. Green. A solution containing 1 mg. (3000 units) per cc. was used.

*Phosphoglucumutase*—In the study of Reaction 2, it was desirable to use a phosphoglucumutase free of both phosphorylase and the enzyme isomerase discovered by Lohmann (21) which catalyzes the equilibrium between glucose-6-phosphate and fructose-6-phosphate. A phosphoglucumutase preparation free of phosphorylase is readily obtained by the treatment of muscle extract with 0.40 saturated ammonium sulfate, which precipitates the phosphorylase practically completely and leaves the phosphoglucumutase in solution. However, this solution still contains a large amount of isomerase, which can then be inactivated by heating at acid reaction, a treatment which does not inactivate phosphoglucumutase.

The enzyme used for the experiments shown in Fig. 1 was prepared as follows: 112 gm. of ammonium sulfate were added at 30° to 400 cc. of a rabbit muscle extract which had been dialyzed for 6 hours; the precipitate was filtered off and discarded. To the filtrate were added 56 gm. of ammonium sulfate. The precipitate was dissolved in 15 cc. of 0.033 M veronal buffer at pH 7.5. The resulting pH was 6.7.

The clear solution was heated for 15 minutes at 52°. A large precipitate was filtered off and the filtrate was dialyzed for 2 hours. A 1:20 dilution of this enzyme solution caused the conversion of added glucose-1-phosphate to the equilibrium mixture of 1- and 6-esters in 10 minutes but still contained isomerase in large amounts, since 39 per cent of the reaction product was fructose-6-phosphate.

The enzyme solution was adjusted to pH 5.2 by addition of 1 cc. of 1.0 M acetate buffer of pH 5.0, and was heated for 30 minutes at 52°. A large precipitate was filtered off, the filtrate was adjusted to pH 7.5 with NaOH, and a second smaller precipitate was removed. A 1:20 dilution of this enzyme caused the conversion of added glucose-1-phosphate to the equilib-

rium mixture of 1- and 6-esters in 15 minutes and was practically free of isomerase, since only 1 per cent of the reaction product was fructose-6-phosphate.

Since heating at neutral reaction did not remove isomerase, this step was omitted in later preparations of phosphoglucomutase. All of the preparations were completely free of diastase and of pyrophosphatase.

*Hexokinase*—In order to demonstrate the formation of polysaccharide from glucose, it was desirable that the hexokinase used to catalyze the reaction between glucose and adenosine triphosphate be free of both phosphatase and pyrophosphatase. Phosphatase interferes by causing the hydrolysis of hexose monophosphate to hexose and inorganic phosphate; pyrophosphatase interferes by causing the hydrolysis of adenosine triphosphate to adenylic acid and inorganic phosphate. The inorganic phosphate formed by both of these reactions makes difficult the estimation of the inorganic phosphate liberation which accompanies polysaccharide formation.

Hexokinase, when prepared according to the procedure of Meyerhof (22),<sup>1</sup> by extraction of autolyzed bakers' yeast with an equal volume of water and precipitation of the extract with an equal volume of alcohol at 0°, still contains the interfering enzymes. It has been found that a precipitate is formed by acidification of the Meyerhof preparation to pH 4.5. The filtrate, which contains all of the hexokinase activity but is almost free of pyrophosphatase, may then be adjusted to pH 6 and fractionated with alcohol. Phosphatase and the remaining traces of pyrophosphatase are precipitated at -2° to -3° by low concentrations of alcohol (15 to 20 per cent). Hexokinase of sufficient purity for these experiments is then precipitated by 25 to 30 per cent alcohol.<sup>2</sup>

The hexokinase used in the experiment shown in Table III was prepared as follows: The alcohol precipitate from 16 liters of yeast extract<sup>3</sup> was suspended in 5 liters of water at 0° and adjusted to pH 4.7 by addition of 300 cc. of 2.0 M acetic acid. A large precipitate, which flocculated when the pH was readjusted to 5.5 with 1.0 N NaOH, was filtered off through fluted paper. The filtrate was precipitated with 25 per cent alcohol at -3°. The low temperature is essential because of the marked increase in the solubility of the protein at higher temperatures. The precipitate was

<sup>1</sup> It was not found necessary to discard the first extract and make a second extraction as suggested by Meyerhof. The two extracts were about equal in their content of both hexokinase and the interfering enzymes cited above.

<sup>2</sup> When the fractionation with alcohol was preceded by salting-out of the protein with ammonium sulfate (followed by dialysis), the alcohol concentrations required were roughly twice as high as before ammonium sulfate treatment.

<sup>3</sup> Kindly supplied by Anheuser-Busch, Inc.

dissolved in cold water (one-tenth of the volume of the original extract). The solution was dialyzed against running cold tap water for 2 hours and precipitated with 21 per cent alcohol at  $-3^{\circ}$ . The precipitate was discarded and the alcohol concentration in the supernatant fluid was increased to 30 per cent. The resulting precipitate was dissolved in water (one-twenty-fifth of the original extract volume). The final solution contained 4.5 mg. of protein per cc. and possessed 5 times as much hexokinase activity per mg. of protein as the original yeast extract. 1 mg. of protein catalyzed the transfer of 0.2 mg. of P per minute at  $30^{\circ}$  and pH 7.5.

Yeast was used as the source of hexokinase in this work because this enzyme has not yet been obtained in highly active form from animal tissues. Crude extracts of brain (23, 24) and heart (25) have been shown to catalyze the reaction between adenosine triphosphate and glucose. The crude animal tissue extracts contain, in addition to the two interfering enzymes mentioned above, a third enzyme which interferes with polysaccharide formation by causing the formation of hexose diphosphate from hexose monophosphate and adenosine triphosphate.

*Myokinase*—Yeast hexokinase alone causes the transfer of only one-half of the labile P of adenosine triphosphate to carbon atom 6 of glucose.<sup>4</sup> However, upon addition of myokinase, a heat-stable protein (26, 27) which occurs in muscle, the hexokinase effects a complete transfer of the labile P to glucose. Since myokinase is active in a concentration of 1  $\gamma$  per cc., it was not surprising to find that the phosphoglucomutase preparation from muscle still contained sufficient myokinase to obviate the necessity of adding it in these experiments.

#### *Preparation of Substrates and Coenzymes*

*Glucose-1-phosphate*—Two samples of synthetic glucose-1-phosphate (28) were used, one crystallized once as the dipotassium salt (29), the other crystallized three times.

*Glucose-6-phosphate*—Since Robison's method (30) of preparing pure glucose-6-phosphate involves a tedious fractionation of the brucine salts of the mixture of fructose- and glucose-6-phosphate isolated from fermenting yeast extract, a simpler method of preparation of the ester from glucose-1-phosphate will be described here.

30 cc. of a reaction mixture (pH 7.4), containing 570 mg. of the dipotassium salt of glucose-1-phosphate, 3 mg. of  $Mg^{++}$ , and 3 cc. of purified phosphoglucomutase, were kept for 15 minutes at  $30^{\circ}$  and deproteinized with 10 cc. of 5 per cent  $HgCl_2$  in 1.0 N HCl. After removal of the mercury

<sup>4</sup> Glucose-1-phosphate is not an intermediate product in this reaction, since the enzyme necessary for its conversion to glucose-6-phosphate is not present (26).

with  $\text{H}_2\text{S}$ , followed by aeration, analysis showed that 92 per cent of the added 1-ester had been converted to the acid-stable 6-ester. The remaining 1-ester was hydrolyzed by heating the acid solution for 5 minutes at  $100^\circ$ . The solution was cooled and neutralized to pH 7.4 with 35 cc. of saturated barium hydroxide. The precipitate of barium phosphate was discarded and the resulting solution (volume 75 cc.) was mixed with 80 cc. of 95 per cent ethyl alcohol to precipitate the barium salt of glucose-6-phosphate. The barium salt was dissolved in water and reprecipitated three times from 50 per cent alcohol, then washed with alcohol and ether, and dried over  $\text{H}_2\text{SO}_4$  *in vacuo* at  $30^\circ$ . The yield of dry barium salt was 302 mg. Analysis for inorganic P showed 0.02 per cent directly, 0.03 per cent after 5 minutes acid hydrolysis, and 7.48 per cent after ashing (calculated for the anhydrous barium salt, 7.85 per cent P).  $[\alpha]_D^{25} = +16.6^\circ$  (for a 2.7 per cent solution of the barium salt in water);  $[\alpha]_D^{25} = +34.2^\circ$  (for a 1.40 per cent solution of the free acid). These values compare well with those calculated from the data of Robison,  $[\alpha]_D^{20} = [\alpha]_{D_{161}}^{20} \times 0.84 = +17.3^\circ$  and  $+34.8^\circ$ , respectively. Analysis for fructose indicated that the product was 1.6 per cent fructose-6-phosphate and 98.4 per cent glucose-6-phosphate.

This preparation was used in the study of the equilibrium constant for the reaction catalyzed by phosphoglucomutase.

*Embden Ester*—For the study of the formation of polysaccharide from glucose-6-phosphate, a mixture (Embden ester), consisting of about 66 per cent glucose-6-phosphate and 34 per cent fructose-6-phosphate, was used. It was prepared from starch and inorganic phosphate by the action of crude dialyzed muscle extract in the presence of small amounts of  $\text{Mg}^{++}$ , adenylic acid, and iodoacetic acid. The product was freed of glucose-1-phosphate by short acid hydrolysis and freed of nucleotides by treatment with mercuric acetate. The final product, isolated as the barium salt, contained 0.08 per cent inorganic P by direct analysis and 7.22 per cent P after ashing. The reducing power, measured with the Shaffer-Somogyi Reagent 50 (20), was 77 per cent of that given by an equivalent quantity of glucose.

Although only the aldose portion of the Embden ester is directly available for polysaccharide formation, the fructose-6-phosphate may also be converted to polysaccharide when isomerase is present in addition to phosphoglucomutase and phosphorylase.

*Adenosine Triphosphate*—This compound was prepared from rabbit muscle by a modification of the method of Lohmann (31). The barium salt was converted to the sodium salt by removing the barium with the stoichiometric amount of sulfuric acid and neutralizing with sodium hydroxide to pH 7.5. The ratio of acid-labile to acid-stable P was 1.9 : 1.

*Glycogen and Adenylic Acid*—For the enzymatic formation of polysaccharide from glucose-1-phosphate it is necessary to add catalytic amounts of glycogen and adenylic acid (14). Glycogen was prepared from dog liver by the method of Somogyi (32). Adenylic acid was prepared by alkaline hydrolysis of adenosine triphosphate according to the method of Lohmann (33).

## Results

### *Equilibrium between Glucose-1-phosphate and Glucose-6-phosphate*

*Position of Equilibrium*—In the experiments illustrated in Fig. 1 the equilibrium was approached from both sides. When glucose-6-phosphate

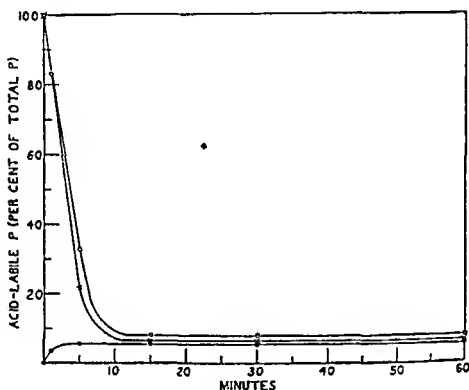


FIG. 1. Equilibrium between glucose-1-phosphate and glucose-6-phosphate at 30°. 1 cc. of reaction mixture contained the potassium salt of the 1-ester or 6-ester (0.4 mg. of P), 0.2 mg. of  $Mn^{++}$ , 0.05 cc. of phosphoglucomutase, and 0.025 M veronal buffer (pH 7.5). ●, glucose-6-phosphate; O, glucose-1-phosphate (crystallized once); X, glucose-1-phosphate (crystallized three times).

was added to the enzyme at 30°, 5.5 per cent was changed to glucose-1-phosphate at equilibrium, as indicated by the appearance of an easily hydrolyzable ester. No inorganic phosphate was liberated. When glucose-1-phosphate, which had been crystallized only once, was added to the enzyme, 8.0 per cent of the labile ester remained when equilibrium was reached. This discrepancy is undoubtedly due to the presence, as an impurity in the glucose-1-phosphate sample used, of 2.5 per cent of an easily hydrolyzable ester which is not acted upon by phosphoglucomutase. This explanation is supported by the fact that, when a sample which had been crystallized three times was used, only 6.5 per cent of the labile ester

remained when equilibrium was reached. It is most likely that the value of 5.5 per cent, obtained from the 6-ester side, is the correct figure.

*Effect of Temperature on Equilibrium*—The equilibrium is shifted slightly toward the side of glucose-1-phosphate as the temperature is raised. Starting from the 6-ester side, one obtains a value of 4.8 per cent 1-ester at 20° and 5.8 per cent 1-ester at 40°. When the once crystallized 1-ester was used, a similar shift with temperature was observed, 7.6 per cent acid-labile P at 20°, 8.4 per cent acid-labile P at 40°.

From the value of the equilibrium constant, one can calculate the free energy change per gm. mole for the conversion of glucose-1-phosphate to glucose-6-phosphate as follows:  $\Delta F^\circ = -RT \ln K = -4.58T \log_{10} K$ . At 30°,  $\Delta F^\circ = -4.58 \times 303 \times \log_{10} (94.5/5.5) = -1720$  calories. Similarly, at 20°,  $\Delta F^\circ = -1740$  calories, and at 40°,  $\Delta F^\circ = -1740$  calories. Since  $\Delta F$  is independent of temperature in this case, it follows from the Gibbs-Helmholtz equation that the heat of reaction,  $\Delta H$ , must be approximately equal to the free energy change.

*Effect of pH on Equilibrium*—The position of equilibrium is not affected by changing the pH. In an experiment in which the three times crystallized 1-ester was used, a value of 6.5 per cent 1-ester at equilibrium at 30° was obtained at pH 6.19 as well as at pH 7.46. The lower pH was obtained by addition of dilute sulfuric acid, the final pH of the reaction mixtures being checked with a glass electrode.

That the equilibrium is independent of pH was to be expected from the fact that the acid dissociation constants for the 1- and 6-esters are identical (28). In the case of the reaction catalyzed by phosphorylase, the position of the equilibrium changes with pH, because the acid dissociation constants of orthophosphate and of glucose-1-phosphate are different (14).

#### *Formation of Polysaccharide from Glucose-6-phosphate*

*Experiments with Crude Muscle Extract*—In studying the formation of polysaccharide from glucose-6-phosphate in dialyzed extracts, it is necessary to add magnesium or manganese ions for the activation of phosphoglucomutase and small amounts of both adenylic acid and glycogen for the activation of phosphorylase. By starting with a system low in inorganic phosphate, one can then observe an appreciable polysaccharide formation; however, by adding barium ions in sufficient concentration to precipitate the inorganic phosphate formed in Reaction 3, a more rapid and complete conversion is made possible. The polysaccharide formed is readily distinguished from the glycogen used to prime the reaction, because the former gives a blue color with iodine, whereas the latter in the concentrations used gives a barely detectable brown color.

In Table I is shown an experiment in which the barium salt of the



Embden ester was added at 30° to a crude muscle extract which had been freed of adenylic acid and inorganic phosphate as described above. It can be seen that when the phosphorylase in the extract was not activated by addition of glycogen and adenylic acid, very little inorganic phosphate was liberated, but 3.8 per cent of the added Embden ester was converted to an acid-labile ester. This corresponds closely to the amount of glucose-1-phosphate to be expected if the glucose-6-phosphate, which comprised two-thirds of the Embden ester, were in equilibrium with the 1-ester ( $\frac{2}{3} \times 5.5 = 3.7$  per cent). When glycogen and adenylic acid were present, inorganic phosphate was liberated to the extent of 5.7 per cent of the added Embden ester in 1 hour. The newly formed polysaccharide gave a purple color with iodine. It will be noted that the concentration of 1-ester was considerably below the phosphoglucomutase equilibrium value, indicating

TABLE I

*Polysaccharide Formation from Embden Ester in Crude Muscle Extract*

0.5 cc of reaction mixture contained the barium salt of the Embden ester (0.662 mg of P), 0.05 mg of  $Mg^{++}$ , 0.3 cc of 0.05 M veronal buffer of pH 7.7, and 0.2 cc of dialyzed muscle extract. Temperature 30°. The results are expressed as per cent of the total P content

Additions	Incubation time	Inorganic P	Acid labile P
	min	per cent	per cent
None . . . . .	0	0.8	0.3
" . . . . .	15	0.9	3.4
" . . . . .	60	1.0	3.8
0.1 mg adenylic acid + 2 mg glycogen	15	2.5	2.2
0.1 " " " + 2 " " "	60	6.5	2.5

that the rate of conversion of the 1-ester to polysaccharide exceeded the rate of formation of the 1-ester from glucose-6-phosphate. In other experiments it was established that neither glycogen nor adenylic acid alone was sufficient to cause a liberation of inorganic phosphate from the Embden ester.

By the use of crude muscle extracts which had been frozen and concentrated 7-fold *in vacuo*, 33 per cent of the added Embden ester could be converted to polysaccharide in 3 hours at 30°, as measured by the increase in inorganic phosphate in the presence of catalytic amounts of glycogen and adenylic acid (Fig. 2). 1 cc. of reaction mixture in these experiments contained roughly the amount of enzyme which can be extracted from 1 gm of muscle; so that the initial rate found corresponds to a formation of about 150 mg. of glycogen per 100 gm. of muscle per hour. A similar calculation for the experiment in Table I with unconcentrated muscle extract gives a

rate of formation of about 400 mg. of glycogen per 100 gm. of muscle per hour.

*Polysaccharide Formation from Embden Ester with Purified Enzymes*—In Table II are shown the results of an experiment in which 26 per cent of the added Embden ester was converted to polysaccharide in 20 minutes at 30° by the action of crystalline phosphorylase plus purified phosphoglucomutase.<sup>5</sup> It can be seen that the amount of polysaccharide formed is in agreement with the amount of inorganic phosphate liberated. Addition of iodine gave a dark blue precipitate with the newly formed polysaccharide. Table II shows that phosphorylase alone had no effect on Embden ester; phosphoglucomutase alone caused no polysaccharide formation, but con-

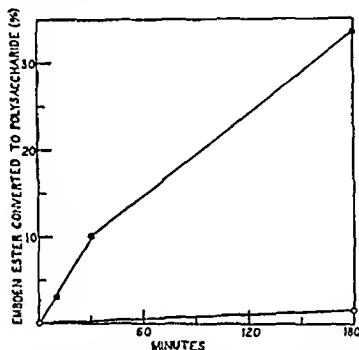


FIG. 2. Polysaccharide formation from the Embden ester in crude muscle extract. The experimental conditions were the same as in Table I, except that the muscle extract was concentrated 7-fold. ●, glycogen and adenylic acid added; ○, glycogen and adenylic acid omitted.

verted part of the Embden ester to glucose-1-phosphate, as indicated by the appearance of a small amount of acid-labile P.

The rate of polysaccharide formation with the purified enzymes was about 500 mg. per 100 cc. of reaction mixture per hour. The concentration of phosphorylase in the reaction mixture (25 mg. per 100 cc.) corresponds roughly to the concentration of phosphorylase in intact muscle (20 to 50 mg. per 100 gm., as estimated from the phosphorylase content of crude muscle extracts).

#### *Synthesis of Polysaccharide from Glucose with Purified Enzymes*

The synthesis was carried out in two steps. In the first step glucose was phosphorylated to glucose-6-phosphate by adenosine triphosphate in the

<sup>5</sup> The rate of the reaction fell off sharply after 20 minutes; this phenomenon, which has been observed repeatedly, has not yet been adequately explained.

presence of purified hexokinase and myokinase. Since purified phosphoglucomutase was also present, about 5 per cent of the glucose-6-phosphate formed was converted to glucose-1-phosphate. After the glucose had been phosphorylated, crystalline phosphorylase, glycogen, and barium acetate were added to bring about the polymerization to polysaccharide.<sup>6</sup>

The original reaction mixture, pH 7.6, consisted of 30 mg. of glucose, adenosine triphosphate (0.25 mg. of inorganic P, 3.26 mg. of acid-labile organic P), 1.8 mg. of hexokinase protein, 14 mg. of phosphoglucomutase (containing myokinase), 0.8 mg. of  $Mg^{++}$ , 1.6 cc. of 0.01 M veronal buffer, and phenol red indicator in a total volume of 2.35 cc. The reaction be-

TABLE II

*Polysaccharide Formation from Embden Ester with Purified Enzymes*

0.4 cc. of a solution, pH 7.2, contained the barium salt of the Embden ester (0.43 mg. of P), 0.8 mg. of adenylic acid, 0.545 mg. of glycogen, 0.08 mg. of  $Mg^{++}$ , 0.1 mg. of crystalline phosphorylase, and 1.7 mg. of purified phosphoglucomutase. When the effect of only one enzyme was to be tested, the other was heated for 5 minutes at 80°. The polysaccharide is expressed as mg. of glucose formed on acid hydrolysis.

Enzymes	Incubation time	Inorganic P	Acid-labile P*	Polysaccharide	Increase in polysaccharide		Color with iodine
					Calculated from increase in inorganic P	Found	
	min.	mg.	mg.	mg.	mg.	mg.	
Phosphorylase + phosphoglucomutase	0	0.004	0.002	0.545			Yellow
Phosphorylase + phosphoglucomutase	20	0.114	0.005	1.199	+0.637	+0.654	Blue
Phosphorylase + phosphoglucomutase	40	0.124	0.009	1.220	+0.696	+0.675	"
Phosphoglucomutase	40	0.005	0.014	0.547	+0.006	+0.002	Yellow
Phosphorylase	40	0.003	0.004	0.533	-0.006	-0.012	"

\* Hydrolysis for 5 minutes in 0.1 N  $H_2SO_4$  at 100°.

came acid to phenol red after less than 1 minute of incubation at 30°, due to the fact that 2 equivalents of acid appear for each mole of adenosine triphosphate which reacts with glucose. Incubation was continued, with gradual addition of 0.15 cc. of 3 per cent sodium hydroxide to maintain a pH of 7.2, for 20 minutes.

<sup>6</sup> Addition of adenylic acid was unnecessary, because large amounts of it are formed in the reaction of adenosine triphosphate with glucose. In the absence of adenylic acid, amorphous preparations of phosphorylase are almost completely inactive, but crystalline phosphorylase exhibits about 60 per cent of its maximal activity.

Aliquots of the 2.5 cc. of reaction mixture were then incubated with phosphorylase, glycogen, and barium acetate. The results are shown in Table III. Analysis before incubation with phosphorylase showed that about 75 per cent of the acid-labile P of adenosine triphosphate had been transferred to glucose, while only 3 per cent was converted to orthophosphate by pyrophosphatase action. Upon incubation with phosphorylase for 20 minutes at 30°, 29 per cent of the hexose monophosphate (or 14 per cent of the added glucose) was converted to polysaccharide, as indicated by inorganic phosphate and polysaccharide analysis, as well as by the blue

TABLE III

*Formation of Polysaccharide from Glucose with Purified Enzymes*

Aliquots of the product of the reaction between adenosine triphosphate and glucose (see the text) were mixed with an equal volume of a solution containing 0.24 per cent glycogen, 3 per cent barium acetate, and 0.05 per cent crystalline phosphorylase. pH 7.2; temperature 30°. The results are calculated per 5 cc. of the final reaction mixture.

Enzyme	Incubation time	Inorganic P	Acid-labile P*	Polysaccharide	Increase in inorganic P due to phosphorylase	Increase in polysaccharide		Color with iodine
						Calculated	Found	
	min	mg.	mg.	mg	mg	mg	mg.	
Phosphorylase	0	0.34	0.76	8.94†				Yellow
"	20	1.08		12.60	+0.70	+4.07	+3.66	Blue
"	40	1.22		13.30	+0.84	+4.88	+4.36	Deep blue
Heated phosphorylase	40	0.38		8.80	0	0	-0.14	Yellow

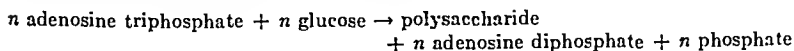
\* Hydrolysis for 10 minutes in 1.0 N H<sub>2</sub>SO<sub>4</sub> at 100°.

† The amount of glycogen added to catalyze the reaction was 6 mg. The additional polysaccharide in the initial sample is due to the presence of 2.9 mg. of a polysaccharide of unknown nature in the hexokinase preparation.

color obtained with iodine. In the control sample incubated with heated phosphorylase, no polysaccharide formation took place.

## DISCUSSION

It is now clear that the energy required to synthesize polysaccharide from glucose is supplied by adenosine triphosphate. The energy is necessary for the phosphorylation of the glucose to glucose-6-phosphate; the conversion to glucose-1-phosphate and the polymerization can then proceed with little change in free energy. One may write the over-all reaction (sum of Reactions 1, 2, and 3) as follows:



where  $n$  is the number of glucose units in the polysaccharide molecule. In the experiment reported here it was necessary to add a large amount of adenosine triphosphate; however, in a respiring system a trace of adenosine triphosphate would be sufficient, because it would be resynthesized continuously from adenosine diphosphate (or adenylic acid) and inorganic phosphate. The oxidation of a wide variety of substrates is known to be "coupled" with the synthesis of adenosine triphosphate in cell-free extracts of animal and plant tissues. Oxidative reactions promote the synthesis of polysaccharide not only by causing the resynthesis of adenosine triphosphate but also by simultaneously lowering the concentration of inorganic phosphate, thus shifting Reaction 1 in the direction of polysaccharide formation, and making unnecessary the addition of a precipitant of inorganic phosphate such as barium.

It is well known that a large number of metabolites, such as lactate, glycerol, and certain of the amino acids, can give rise to glycogen formation in animal tissues. It appears probable that in all these cases there is a final common pathway leading from glucose-6-phosphate to glycogen. Glucose-6-phosphate may be formed from phosphopyruvic acid by a reversal of the reactions of the Embden-Meyerhof scheme. However, the direct conversion of pyruvic acid (formed from lactic acid and other substances) into phosphopyruvic acid has not yet been experimentally demonstrated. Recent work by Hastings and coworkers (34) and others indicates that this step in polysaccharide synthesis may involve an uptake of carbon dioxide, leading to the formation of  $C_4$ -dicarboxylic acids which upon oxidation give rise to phosphopyruvic acid (35).

#### SUMMARY

1. The enzyme phosphoglucumutase, which catalyzes the reversible reaction, glucose-1-phosphate  $\rightleftharpoons$  glucose-6-phosphate, has been prepared free from other enzymes which act on these two substances, by an ammonium sulfate fractionation of muscle extract, followed by a heat treatment at acid reaction.

2. Pure glucose-6-phosphate has been prepared by the action of the above enzyme on glucose-1-phosphate. Both esters are converted by phosphoglucumutase to the same equilibrium mixture.

3. At equilibrium, 4.8 per cent of the glucose monophosphate is present as 1-ester at 20°, 5.5 per cent at 30°, and 5.8 per cent at 40°. The position of equilibrium is independent of pH.

4. By the combined action of phosphoglucumutase and phosphorylase, a rapid formation of polysaccharide from glucose-6-phosphate has been obtained (500 mg. per 100 gm. of muscle per hour). Polysaccharide and inorganic phosphate are formed in equivalent amounts.

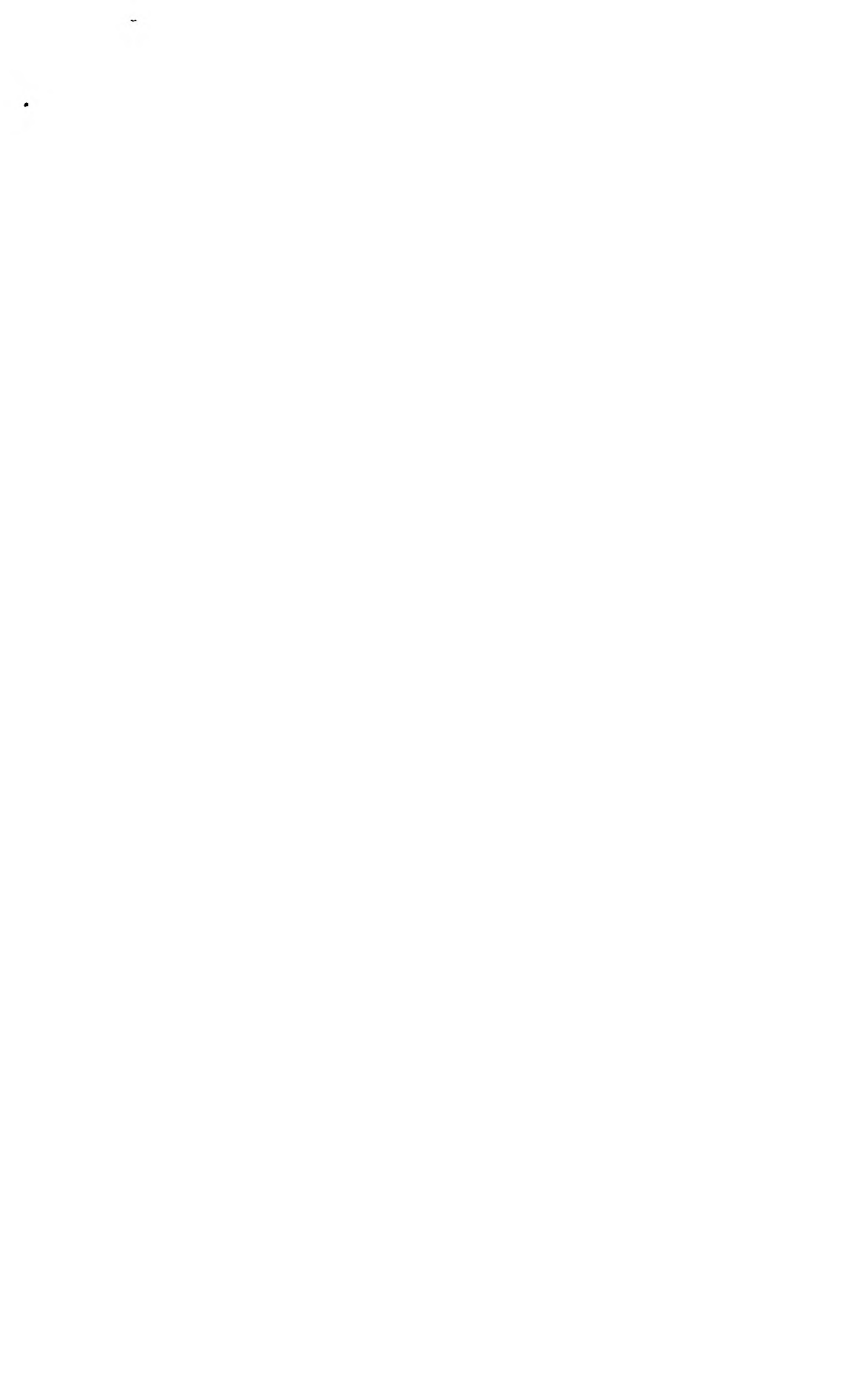
5. Yeast hexokinase, which catalyzes the formation of glucose-6-phos-

phate from glucose and adenosine triphosphate, has been purified by acid treatment, followed by fractionation with alcohol, and a rapid synthesis of polysaccharide from glucose has been effected by the successive action of hexokinase, phosphoglucomutase, and phosphorylase.

The authors are grateful to Professor Carl F. Cori for his continued interest in this work.

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# OCCURRENCE OF SPHINGOMYELIN IN TISSUES OF THE CAT\*

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Although the presence in tissues of at least three different phospholipids has been known for many years, the methods available limited earlier studies chiefly to the total phospholipid fraction in different tissues. Only in recent years have detailed studies concerning the lecithins and cephalins appeared and added to our knowledge of their possible functions. Considerably less is known concerning the phospholipid sphingomyelin.

The present investigation was undertaken for the purpose of obtaining more information concerning the distribution of sphingomyelin and with the hope that these data might furnish some clue as to its function. While this work was in progress, several reports of closely related studies appeared, particularly those of Thannhauser and coworkers (1, 2), of Ramsey and Stewart (3), and of Erickson *et al.* (4). These investigations have made available additional data for comparison with the analyses reported in this paper.

## EXPERIMENTAL

*Animals*—Eleven female cats were used, but values for all of the different tissues are not always available for each animal. The animals weighed from 2.62 to 3.52 kilos, with an average of 2.88. They were maintained on a diet of scraps from the hospital kitchens.

*Treatment of Tissues*—The animals were lightly anesthetized with ether and bled from the jugular veins. In some cases the blood was collected with solid sodium citrate as anticoagulant. The blood was centrifuged and the plasma drawn off into 95 per cent ethyl alcohol. The cells were washed once with isotonic saline and the washing discarded.

The solid tissues (brain, kidney, heart, spleen, lung, intestine, liver, and skeletal muscle from the thigh) were excised immediately after the death of the animal. The organs were freed of visible fat, and in the case of skeletal muscle the large nerves were removed. The intestinal mucosa was separated from the intestinal muscle by the technique of Sinclair (5).

\* These studies are taken from a dissertation submitted to The University of Rochester in partial fulfilment of the requirements for the degree of Doctor of Philosophy, June, 1941.

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*Extraction of Lipids*—The tissue, after it had been ground with sand, was heated on the steam bath with the solvents described in the preliminary report (6). The necessity for using 1:1 methanol-chloroform for the complete extraction of sphingomyelin, originally used by the author because Thannhauser and Setz (7) found it necessary for complete extraction of dried tissues, has been verified for wet tissue by Haven and Levy (8). The blood proteins, precipitated when the plasma was added to alcohol, were subjected to further lipid extractions.

For the reextraction of the lipids from the residue remaining after removal of the original solvents under diminished pressure petroleum ether containing 5 to 10 per cent chloroform was used (6). While this study was in progress, Erickson *et al.* (4) published a report in which they refer to unpublished work which indicates that sphingomyelin may not be quantitatively reextracted by petroleum ether. Work in this laboratory by Haven and Levy (8) and by Taylor (9) has indicated that sphingomyelin, while relatively insoluble in petroleum ether when pure, is completely extracted when in a mixture of lipids. More extensive studies may be needed to clear up this point.

*Total Phospholipid Determination*—An aliquot of the petroleum ether-chloroform extract was used for isolating the phospholipids as described by Bloor (10). The phospholipids were not measured oxidatively, but phosphorus determinations were made and the phospholipid calculated by multiplying the phosphorus values by 25.

*Sphingomyelin Determinations*—A micro modification (6) of Thannhauser's method (7) with Reinecke salt was used. The principal differences from the modification published by Erickson *et al.* (4) are the use of somewhat larger quantities and of centrifugation instead of the sintered glass filter sticks for the separation and washing of the precipitate. Haven and Levy (8) have used essentially the same procedure for gravimetric determinations.

Not all commercial samples of Reinecke salt were satisfactory for use. Some gave little or no precipitate with sphingomyelin, while others gave a greenish or gray precipitate instead of the pink crystalline material described by Thannhauser and Setz (7). Eimer and Amend Reinecke salt was usually satisfactory. When this could not be obtained, satisfactory material was synthesized (11) in the laboratory. An additional recrystallization was added to the procedure.

One detail in the washing of the sphingomyelin reineckate merits some discussion. A cold acetone washing was included by Thannhauser and coworkers (1), because it was necessary for the removal of some phosphorus-containing substance, originally thought to be a form of sphingomyelin (7), but later considered as an impurity. The author has found

that the acetone-soluble fraction may contain very appreciable amounts of phosphorus. Because of this finding two cold acetone washings were included in each determination reported here. This acetone-soluble reineckate was nearly absent in some determinations, but present in relatively large amounts in determinations on extracts from liver and skeletal muscle. Erickson *et al.* (4) give several values which indicate that washing with acetone did not remove any phosphorus. If the tests were conducted on a purified sphingomyelin preparation or on phospholipids from certain tissues, it is easy to understand that such results would be obtained.

For the calculation of the amount of sphingomyelin the value for phosphorus in the reineckate was multiplied by a factor of 26. This factor, arbitrary by necessity, makes some allowance for the fact that almost all purified sphingomyelin samples fall below 4 per cent in phosphorus content, and for the fact that sphingomyelin may contain an extra fatty acid (2).

*Phosphorus Determinations*—These were carried out according to the method of Kuttner and Lichtenstein (12).

*Accuracy of Method*—Erickson and coworkers (4) have reported recoveries of 76 to 113 per cent on known amounts of "purified" sphingomyelin. The sphingomyelin preparations were 70 to 90 per cent pure. When sphingomyelin was added to stroma extracts, the recoveries ranged from 97 to 110 per cent. The average for the whole series was 98 per cent with a standard deviation of  $\pm 8.8$ .

The author has not carried out an extensive series of analyses, but six determinations on highly purified sphingomyelin gave recovery values of 90, 90.6, 89.8, 93.3, 103, and 93.9 per cent. In a series of twenty tissue analyses performed in duplicate the individual values had a standard deviation from the means of  $\pm 10.1$  per cent. Taylor (9) has reported 95 per cent recovery of purified sphingomyelin.

### Results

The data obtained for the different tissues are recorded in Table I. In cases in which it was possible duplicate determinations were made on each sample, but with many tissues (heart, spleen, skeletal muscle, blood plasma, and blood cells) the amount of material was insufficient for more than one determination. The concentration of sphingomyelin is based on the wet weight of the tissue.

### DISCUSSION

A survey of the literature yields little information on the occurrence of sphingomyelin. Thannhauser and coworkers (1) have published some values for human tissues. Erickson *et al.* (4) have presented a few values

for brain and blood. Reports on ether-insoluble phospholipids in blood have been published by Artom and Freeman (13) and Kirk (14), while Ramsey and Stewart (3) have used an entirely new method to obtain values for blood. None of these studies has been carried out on cats.

*Brain*—The values for sphingomyelin in the brain tissue of cats (Table I) agree fairly well with those in the literature for other species and very closely with those found by Taylor for cats (9).

*Heart and Spleen*—The data for these tissues correspond roughly to similar values reported by Thannhauser *et al.* (1) for human tissues.

*Kidney, Lung, and Liver*—Only one report of differential phospholipid analyses including sphingomyelin determinations has been published. Thannhauser *et al.* (1) record analyses on samples from three normal

TABLE I  
*Phospholipid and Sphingomyelin in Cat Tissues*

The values are the averages followed by their standard deviations.

Tissue	Total phospholipid		Sphingomyelin		Per cent sphingomyelin in total phospholipid
	No. of samples	Per cent of wet weight	No. of samples	Per cent of wet weight	
Brain . . . . .	10	5.34 $\pm$ 0.42	9	1.25 $\pm$ 0.19	23.6 $\pm$ 3.9
Lung . . . . .	10	2.08 $\pm$ 0.37	11	0.69 $\pm$ 0.12	33.2 $\pm$ 4.7
Kidney ..	11	2.70 $\pm$ 0.44	9	0.48 $\pm$ 0.09	18.7 $\pm$ 3.2
Spleen			11	0.33 $\pm$ 0.09	
Intestinal mucosa	9	1.40 $\pm$ 0.21	7	0.32 $\pm$ 0.06	22.7 $\pm$ 6.0
Liver	8	3.14 $\pm$ 0.36	8	0.23 $\pm$ 0.05	7.5 $\pm$ 2.0
Intestinal muscle	11	0.72 $\pm$ 0.08	11	0.19 $\pm$ 0.03	27.1 $\pm$ 3.4
Heart	9	1.96 $\pm$ 0.28	10	0.15 $\pm$ 0.05	7.8 $\pm$ 1.9
Skeletal muscle	9	0.80 $\pm$ 0.10	9	0.075 $\pm$ 0.011	9.5 $\pm$ 1.6
Blood cells	4	0.490 $\pm$ 0.034	8	0.123 $\pm$ 0.022	26.5 $\pm$ 2.7
" plasma	4	0.177 $\pm$ 0.021	5	0.027 $\pm$ 0.011	14.9 $\pm$ 5.8

humans. The values for the corresponding tissues from cats (Table I), if calculated to a dry weight basis, would be appreciably higher than the values reported for human tissues. In the case of kidney and liver the difference is approximately 50 per cent, while the sphingomyelin concentration in cat lung tissue would appear to be nearly twice that in human lung. Further work will be necessary to confirm these differences or to make apparent the reason for such discrepancies.

*Intestinal Mucosa, Intestinal Muscle, and Skeletal Muscle*—Previous reports have not included investigations with respect to the sphingomyelin content of these tissues.

*Blood Plasma and Blood Corpuscles*—The values for blood plasma correspond rather closely with those reported for human serum by Thann-

hauser *et al.* (1) and for human plasma by Erickson *et al.* (4). Kirk (14) and Artom and Freeman (13) report values obtained by another method, but in both cases there is such a wide range of values (17 to 150 mg. and 13 to 75 mg. per 100 cc.) that averages mean little. The five values on which the figure in Table I is based were 34, 37, 9, 20, and 34 mg. per 100 cc.

Ramsey and Stewart (3) have calculated the amount of the different phospholipids present in whole blood on the basis of phosphorus, choline, and glycerol determinations. The values for sphingomyelin are unusually high (56 to 70 per cent of the total phospholipid), while lecithin would appear to represent only a very small part of the blood phospholipids. The fact that sphingomyelin is calculated by difference is not sufficient to explain the high values if the glycerol method is as accurate as the authors feel it is. It is assumed that all phospholipids not containing glycerol will be sphingomyelins. If part of the blood cephalin did not have glycerol in the molecule (perhaps inositol instead (15)), the calculations would result in low lecithin and high sphingomyelin values. Low choline recoveries due to incomplete hydrolysis would cause low lecithin and high cephalin values, but no change in sphingomyelin.

The values in Table I for total phospholipid and sphingomyelin in the blood cells are higher than reported for other species (14, 4, 16). However, in view of the large species differences in phospholipid in the red blood cell found by Erickson *et al.* (16), one should avoid comparisons between species. The reports for different species agree fairly well concerning the relation of sphingomyelin to total phospholipids.

*General Discussion*—The tissues investigated fall into the following order with respect to sphingomyelin concentration (wet weight basis), brain, lung, kidney, spleen, intestinal mucosa, liver, intestinal muscle, heart, blood corpuscles, skeletal muscle, blood plasma. There is no correlation between the total phospholipid and the amount of sphingomyelin in different tissues. This fact would tend to indicate that sphingomyelin has a different function from that of the other phospholipids.

According to the author's figures the amount of sphingomyelin in the blood plasma is not sufficient for it to play any considerable part in the transport of fatty acids, but Ramsey and Stewart (3) have reported much higher values. The source of plasma sphingomyelin cannot be established with certainty. Some data (unpublished) on the incorporation of radioactive phosphorus into plasma sphingomyelin indicate that both the liver and the intestinal mucosa may contribute sphingomyelin to plasma. The suggestion by Thannhauser and Reichel (2) that sphingomyelin may carry an additional fatty acid would make it possible for it to take part in the transport of fatty acids through the mucosa.

If the phospholipids of the liver are considered especially active in fat

metabolism, it would appear that sphingomyelin is not particularly necessary for this process, for in that tissue sphingomyelin constitutes the smallest percentage of the total phospholipids.

There is rather good evidence that sphingomyelin is associated with the structural portion of the phospholipids in certain cases. Erickson *et al.* (16) found that nearly all of the phospholipid of the red blood cell stays with the stroma on hemolysis and resists washing out.

Because of the large amounts of sphingomyelin in nervous tissue, it is natural to consider the possibilities (1) that its concentration in a tissue may bear a relation to the nervous structures in that tissue or (2) that its concentration may represent a degree of specialization in properties similar to nervous tissue. Present information on these points is inadequate. The high concentration of sphingomyelin in the lung, where it represents about one-third of the phospholipid, is an interesting finding. Studies which reveal the rôle of sphingomyelin in lung tissue may explain one of its functions in other tissues.

#### SUMMARY

The sphingomyelin concentration found in eleven tissues from cats varied from 0.075 per cent in skeletal muscle to 1.25 per cent in the brain. The proportion of sphingomyelin in the total phospholipid ranged from 7.5 to 33.2 per cent; so that there was no correlation between sphingomyelin and total phospholipid.

The values are compared to those found for tissues from other species by previous workers.

Some possible relationships of sphingomyelin to fat metabolism and special properties or functions of tissues are discussed.

The author wishes to express his sincere appreciation to Professor W. R. Bloor and to many members of his department for helpful criticism and advice.

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# MEDICAL EVALUATION OF NUTRITIONAL STATUS\*

## XII THE STABILITY OF ASCORBIC ACID IN WHOLE BLOOD, PLASMA AND PLASMA FILTRATES

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In the course of a study of the nutritional status of a large group of school children (1) determinations of the fasting level of plasma ascorbic acid were performed. Since it was necessary to draw the blood specimens at a public high school and transport them to the laboratory, some time necessarily elapsed before the analyses were performed. It was therefore important to know whether any loss of ascorbic acid occurred during this interval of time.

There is no general agreement in the literature on the question of the stability of ascorbic acid in whole blood, or in its derivatives, plasma and plasma filtrates. Borsook *et al.* (2) found that ascorbic acid remains in the reduced state much longer in whole blood than in plasma and they attribute this stabilizing effect of the cells to the presence of glutathione. Barron, Barron, and Klemperer (3) likewise pointed out that fluids of animal origin containing glutathione possess mechanisms inhibiting the oxidation of ascorbic acid.

To prevent the loss of ascorbic acid in drawn blood, Pijoan and Klemperer (4) and Pijoan and Eddy (5) suggested the use of potassium cyanide (1 to 2 mg. per cc. of blood). Mindlin and Butler (6) also employed potassium cyanide for the same purpose.<sup>1</sup> It has since been shown by several investigators (7-10) that potassium cyanide employed in this manner does not exert any uniform protective action on ascorbic acid and may in some instances invalidate the results because of an apparent enhancement of the

\* This paper is one of a series from a cooperative investigation by the Cornell University Medical College, Department of Public Health and Preventive Medicine and Department of Pediatrics; the Milbank Memorial Fund; the Department of Health of the City of New York; and the United States Public Health Service, National Institute of Health, Division of Public Health Methods. The cooperating agencies have been assisted in carrying out this investigation by the Work Projects Administration for the City of New York, Official Project No. 65-1-97-21, W. P. 24, "Medical evaluation of nutritional status." Other papers in this series are published in the *Milbank Memorial Fund Quarterly* for 1940, 1941, and 1942.

<sup>1</sup> These authors have since abandoned the use of potassium cyanide (personal communication).



ascorbic acid values. Cushman and Butler (9) showed that reduced ascorbic acid is stable in whole blood and metaphosphoric acid filtrates for as long as 24 hours (6 hours at room temperature and 18 hours refrigerated) but that there is a significant loss when plasma stands at room temperature for more than 4 hours. These observations were limited to two specimens of blood. More recently Kassan and Roe (11) reported observations in which the ascorbic acid content of blood plasma kept in contact with the red cells remained unaltered for 16 to 24 hours at room temperature and for 52 hours at refrigerator temperature. The ascorbic acid content of separated plasma decreased on standing. These authors also pointed out the importance of preventing hemolysis and suggested the use of Pyrex, paraffin- and collodion-lined tubes as the best containers for storage.

In the present study we have investigated on a large series of blood specimens the stability of plasma ascorbic acid when left in contact with the red cells, when separated, and in metaphosphoric acid filtrates.

#### EXPERIMENTAL

The method of Mindlin and Butler (6) was used for the determination of ascorbic acid in the plasma separated from oxalated blood.<sup>2</sup> In order to determine whether any changes observed in the ascorbic acid content were greater than the usual experimental error we first evaluated the reliability of the method in our hands. This was done in two ways, (1) by determining the reproducibility obtainable in duplicate determinations on the same specimen and (2) by determining the recovery of ascorbic acid added to the plasma. The latter test was performed by dissolving a known quantity of the crystalline material in the 5 per cent metaphosphoric acid solution used for the precipitation of the plasma proteins. A quantity of ascorbic acid equivalent to 1 mg. per 100 ml. of plasma was uniformly added to this series. The results of these observations are recorded in Table I. These data indicate that both criteria of reliability have been met; namely, excellent agreement between duplicates and complete recovery of added ascorbic acid.

For the study of the stability of ascorbic acid in whole blood the subjects were brought into the laboratory, so that as little time as possible elapsed between the drawing of the blood and the preparation of the first centrifugate. The blood was delivered into an oxalated vessel and after being mixed was divided into three or four equal portions contained in tightly stoppered centrifuge tubes. In one series the tubes were kept at refrigerator temperature (5-8°). In another they were kept at room temperature (approximately 25°). One portion was centrifuged immediately, followed

<sup>2</sup> The metaphosphoric acid-protein precipitate was removed by centrifugation.

by separation of the plasma and precipitation of the plasma proteins. The other portions were centrifuged at intervals of 3 to 4 hours and 24 hours for the refrigerated samples and at intervals of 3, 6, and 24 hours for the series kept at room temperature. In all cases after separation of the plasma the plasma proteins were precipitated with metaphosphoric acid without further delay.

To study the stability of the ascorbic acid in plasma, the entire sample of blood was first centrifuged and the plasma obtained. This was then subdivided into three equal portions, one of which was immediately deproteinized and the others stored in tightly stoppered tubes.

TABLE I

*Reliability of Method for Determination of Ascorbic Acid*

The values are given in mg. per cent.

Duplicate determinations; protein ppt. removed by centrifugation (150 cases)		1 mg. per cent ascorbic acid added to plasma-protein ppt. removed by centrifugation (82 cases)	
Mean value of determinations (I)	0.5730	Mean value before addition	0.619
Mean value of determinations (II)	0.5745	Mean value after addition	1.622
Difference between means (II - I)	$0.0015 \pm 0.0036$	Average recovery	100%
Difference between duplicates with signs, s.d.*	0.044		

\* From duplicate determinations on each specimen, the difference between the two determinations was taken in a constant order (II - I) and the sign of the difference retained. These differences afford a frequency distribution for which the standard deviation was computed in the usual manner; that is, the standard deviation =  $\sqrt{\sum d^2 / (n - 1)}$  and  $d$  is the deviation of the difference value from the mean of the differences with signs and  $n$  is the number of difference values. The mean of the differences is equal to the difference between the mean value of determinations (I) and the mean value of determinations (II) (12).

Finally, to determine the stability of ascorbic acid in metaphosphoric acid centrifugates, three independent centrifugates were prepared simultaneously from the sample of plasma. The ascorbic acid of one of these centrifugates was immediately determined in the colorimeter; the others were kept in tightly stoppered tubes for 3 to 4 hours and 24 hours.

The data of these experiments are presented in Table II. There is no significant change in the plasma ascorbic acid when the plasma is left in contact with the blood cells and stored at refrigerator temperature up to 24 hours. At room temperature the change within 3 hours is insignificant, but at 6 hours the decrease, though slight, is significant and at 24 hours is appreciable.

When the metaphosphoric acid-protein precipitate is removed by filtration, a significant loss of ascorbic acid occurs. This loss is obviated by centrifugation.

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# THE INACTIVATION OF PYRIDINE NUCLEOTIDES BY ANIMAL TISSUES IN VITRO\*

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It has been demonstrated that the inactivation of the pyridine nucleotides by animal tissues *in vitro* is accomplished by the cleavage of nicotinamide from the remainder of the nucleotide molecule (1). Schlenk has found that nicotinamide nucleoside is one of the end-products of the decomposition of cozymase (diphosphopyridine nucleotide; DPN) by an enzyme system present in preparations of sweet almond emulsin (2). Further,

TABLE I

*Effect of Incubation with Broken Cell Preparation of Brain on V Factor Activity of 40  $\gamma$  of DPN and 75  $\gamma$  of Nicotinamide Nucleoside*

The V factor activity is expressed as galvanometer units of growth of *Hemophilus parainfluenzae* when the suspension was properly diluted and added to the proteose-peptone medium

Suspension tested	Galvanometer units
Brain preparation .	0
DPN. .	22
" + brain	0
Nicotinamide nucleoside .	16.5
" " + brain	16.5

nicotinamide nucleoside can serve as V factor (3). Since animal tissues destroy the V factor activity of the pyridine nucleotides (1), it was concluded that nicotinamide nucleoside could not be the end-product of this decomposition. It was also postulated that nicotinamide nucleoside was not an intermediate in this process. The following experiments further support this hypothesis.

75 per cent nicotinamide nucleoside was generously supplied by Dr. F. Schlenk. The procedure used was identical with that previously described (1). 40  $\gamma$  of DPN and 75  $\gamma$  of nicotinamide nucleoside were each incubated with 0.5 cc. of an undiluted broken cell preparation of rat brain for 30 min-

\* Our thanks are due to the John and Mary R. Markle Foundation for support of this study.

The absence of radioactivity from the residual glycerophosphoric acid isolated in the enzyme experiments, which were stopped before the hydrolysis was complete, demonstrates that under the experimental conditions no appreciable synthesis of the phosphoric acid ester from glycerol and inorganic phosphate took place and that the enzymatic attack did not labilize the substrate to such an extent as to induce free exchange with the inorganic phosphate of the medium.

#### EXPERIMENTAL

*Methods*—The preparations were tested for radioactivity in the dry state by means of a Geiger-Müller counter according to methods previously described (7, 8). Standard measurements, accompanying each set of determinations, were carried out with an intimate mixture of small amounts of radioactive sodium phosphate with inactive barium glycerophosphate.

*Conversion of  $\beta$ - into  $\alpha$ -Glycerophosphoric Acid*—To a solution of 5.00 gm. of crystalline sodium  $\beta$ -glycerophosphate (containing  $5\frac{1}{2}$  molecules of water of crystallization) in 50 cc. of water 0.8 mg. of sodium phosphate containing 4 microcuries of  $P_{15}^{32}$  and 5 cc. of concentrated sulfuric acid were added. The solution was kept boiling with a reflux for  $\frac{1}{2}$  hour and worked up for sodium glycerophosphate according to the method of Verkade *et al.* (5). The total amount of the crude salt obtained was dissolved in 150 cc. of water, 20 cc. of a 10 per cent solution of phosphoric acid in water were added, and the mixture was made alkaline with barium hydroxide. The precipitate was removed by filtration through celite and the removal by dilution of radioactive inorganic phosphate repeated twice by the addition and the precipitation of phosphoric acid in the amounts indicated above. The filtrate from the barium phosphate was, after removal of the excess barium by means of  $CO_2$ , concentrated *in vacuo* to a volume of 70 cc. and barium glycerophosphate precipitated by the addition of 270 cc. of absolute alcohol. The amorphous barium salt, which weighed 3.5 gm., showed practically no radioactivity. The crystalline barium  $\alpha$ -glycerophosphate was isolated according to the method of Fischer and Pfähler (9). After two recrystallizations from large amounts of water (9) 1.69 gm. of crystalline barium  $\alpha$ -glycerophosphate were obtained. This salt which was completely devoid of radioactivity was found by means of lead tetraacetate (10) to contain 93.7 per cent of the  $\alpha$  form.

$C_3H_7O_4PBa$  (307.4). Calculated, P 10.1, Ba 44.7; found, P 9.9, Ba 44.3

*Hydrolysis of  $\beta$ -Glycerophosphoric Acid by Kidney Phosphatase in Presence of Radioactive Phosphate*—In these experiments a phosphatase preparation from pig kidneys, obtained by the method of Albers and Albers (11), was employed. The experimental results are summarized in Table I. The

substrate (sodium  $\beta$ -glycerophosphate containing  $5\frac{1}{2}$  molecules of water of crystallization) and the radioactive sodium phosphate were dissolved in glycine buffer of pH 9.4 to give a total volume of 25 cc. and the solution was, in some cases after the addition of small amounts of glycerol, subjected to the action of the enzyme at  $37^{\circ}$ . Control experiments in which the enzyme was omitted were run simultaneously. The degree of hydrolysis of the phosphoric acid ester was followed by the colorimetric determination of inorganic P at regular intervals. After exactly 2 hours the experiments were stopped and 20 cc. of 10 per cent phosphoric acid added. The inorganic phosphate was removed by means of  $\text{Ba}(\text{OH})_2$  and this dilution process (addition of phosphoric acid and precipitation with  $\text{Ba}(\text{OH})_2$ ) repeated twice. The final filtrates from the barium phosphate were neutralized with

TABLE I  
*Enzymatic Hydrolysis of Sodium  $\beta$ -Glycerophosphate*

Experiment No.	Sodium $\beta$ -glycerophosphate used	Radioactive sodium phosphate*	Glycerol	Kidney phosphatase	Sodium $\beta$ -glycerophosphate hydrolyzed after 2 hrs.	Radioactivity of barium nitrate complex of barium $\beta$ -glycerophosphate isolated
	mg.	mg. P	mg.	mg.	per cent of initial amount	KF units in 1 mg. P
1a	802.1	8.5 (3,700)	0	0	0	0
1b	803.8	8.5 (3,700)	0	9.9	25.2	0
1c	800.8	8.5 (3,700)	250	10.2	24.5	0
2a	800.9	6.8 (21,000)	0	0	0	33
2b	800.5	6.8 (21,000)	0	10.0	46.5	42
2c	800.8	6.8 (21,000)	250	10.0	45.5	51

\* The figures in parentheses indicate the radioactivity in KF units (8) per 1 mg. of P.

$\text{CO}_2$ , freed of  $\text{BaCO}_3$ , acidified with acetic acid, and concentrated *in vacuo* to a volume of 10 cc. Barium glycerophosphate was precipitated by the addition of alcohol and in all experiments found to be practically free of radioactivity. From these barium salts the barium nitrate double salts of barium  $\beta$ -glycerophosphate (12) were prepared subsequently. Table I shows that these complexes likewise exhibited no radioactivity in Experiment 1 and only very slight radioactivity (less than 0.25 per cent) in Experiment 2.

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## SUMMARY

The conversion of  $\beta$ -glycerophosphoric acid into the  $\alpha$  form in acidic solution proceeds in the presence of radioactive sodium phosphate without an exchange between the phosphoric acid ester and the inorganic phosphate. The hydrolysis of  $\beta$ -glycerophosphoric acid by kidney phosphatase in the presence of radioactive sodium phosphate likewise takes place without labilization of the phosphoric acid ester linkage.

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# THE MOLECULAR WEIGHT OF INSULIN

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In some of the earliest work with the ultracentrifuge, Sjögren and Svedberg (1) found by the equilibrium method that crystalline insulin appeared to have a molecular weight of 35,100. It has been suggested recently by Svedberg and Pedersen (2), however, that this value is probably too low, since insufficient time was allowed for the attainment of complete equilibrium. A second value for the molecular weight, namely 40,900, has been estimated by Polson (3) on the basis of a diffusion rate,  $D_{20} = 8.20 \times 10^{-7}$  sq. cm. per second, obtained by him, and the sedimentation rate,  $s_{20} = 3.47 \times 10^{-13}$  cm. per second per unit centrifugal field, reported by Sjögren and Svedberg (1). The sample of insulin used for the diffusion measurements was a commercial preparation, the homogeneity of which had been improved by means of fractionation,<sup>1</sup> but which still may have been somewhat impure. Because of the discrepancy between the two molecular weight values reported and the uncertainties which were attached to the measurements in each case, it was thought of importance to repeat certain of the determinations and to employ carefully recrystallized protein for the entire investigation.<sup>2</sup>

*Source of Insulin*—In order to obtain insulin protein of a sufficient degree of homogeneity, commercial samples of crystalline insulin<sup>3</sup> were recrystallized by the method described by du Vigneaud, Miller, and Rodden (5). For the diffusion experiments, two recrystallizations were found to be advisable.

The insulin solutions were prepared as illustrated by the following exam-

\* Rockefeller Fellow, Upsala, Sweden. The recipient of the fellowship wishes to acknowledge his indebtedness to Professor Vincent du Vigneaud through whose kindness the arrangements with Professor The Svedberg were made for the carrying out of the present investigations.

<sup>1</sup> Andersson, K. J. I., unpublished results, quoted by Svedberg and Pedersen (2).

<sup>2</sup> The authors wish to thank sincerely Professor The Svedberg for the privilege of carrying out this and other studies (4) in his laboratory. Thanks are also due Dr. W. M. Stanley and Dr. Max A. Lauffer of the Rockefeller Institute for permission to use the diffusion set-up in their laboratory for a part of the present investigation.

<sup>3</sup> The insulin used in the present study was a sample ofletin generously put at our disposal by Professor du Vigneaud.



ple. 100 mg. of recrystallized insulin were suspended in 5 cc. of water and dissolved by means of 0.9 cc. of 0.1 N hydrochloric acid, added 0.1 cc. at a time with stirring. 1.0 cc. of 0.1 M phosphate buffer at pH 7.1 was then added, followed by 1.1 cc. of 0.1 M sodium hydroxide, added 0.1 cc. at a time with stirring. 1.0 cc. of 2.0 M sodium chloride and 1.0 cc. of water were finally added. The resulting solution, the pH of which was between 7.0 and 7.3, was slightly opalescent owing to the presence of small amounts of impurities. By centrifugation at 5° in a Swedish angle centrifuge, a water-clear supernatant solution was obtained. Before use in diffusion experiments, the protein solutions were dialyzed overnight against a buffer of similar composition.

*Sedimentation Constant of Insulin*—Determinations of the sedimentation constant of insulin were made in the oil-driven ultracentrifuge at a speed of 65,000 R.P.M., corresponding to a centrifugal force of 350,000 times gravity. The observations of the sedimenting boundaries were made by the scale method of Lamm (6). A series of sedimentation runs with solutions containing 1 per cent crystalline insulin in 0.2 M sodium chloride and 0.04 M phosphate buffer at pH 7 gave an average  $s_{20}$  of  $3.55 \times 10^{-13}$  cm. per second per unit centrifugal field. The variation from the mean was very small. The figure given has been corrected for the density and viscosity of the medium, as is now the general practice (2), and is for this reason slightly higher than the value previously reported.

Sjögren and Svedberg (1) found that crystalline insulin was stable within the pH range of approximately 4.5 to 7. Outside these limits, the sedimentation constant of the protein dropped, apparently owing to a dissociation of the insulin. In this earlier work, the photographs of the sedimenting boundaries were obtained by the light absorption method. Since the refractometric method of Lamm provides a more descriptive picture of the process of sedimentation, it was thought of interest to employ this method in making a few investigations on the crystalline insulin outside its stability range. Two sedimentation runs were therefore made, one at pH 7.5, the other at pH 8.5. The sedimentation diagrams for these experiments are shown in Fig. 1. It may be seen that at pH 7.5 the insulin was quite stable and yielded the normal sedimentation constant. At pH 8.5, however, the rate of sedimentation fell considerably, and in addition there was a progressive dissociation in the direction of the formation of smaller and smaller particles. It may be concluded that, in general, the results of the present sedimentation studies are in close agreement with the corresponding data of Sjögren and Svedberg.

*Diffusion Constant of Insulin*—Diffusion measurements on the insulin were made at 20° in the metal diffusion cell and by the refractometric method as described by Lamm (6), and also at 0° with the Neurath diffu-

sion cell (7) and optical system of Longworth (8). The experiments were carried out on concentrations of protein varying from 0.5 to 1.0 per cent. The solvent used was 0.2 *N* NaCl in 0.01 to 0.02 *M* phosphate buffer at pH 7. The diffusion constants for the measurements determined with the Lamm apparatus were calculated by the method of "successive analysis." The results obtained with the Neurath-Longworth apparatus were calculated by the "maximum ordinate-area" method and also by the method based on the measurements of the abscissas for the inflection points of the concentration gradient curves. These methods of calculation have all been described by Lamm (6). For the diffusion curves which were obtained by the Longworth scanning method, the Lamm equations were applied as follows, as suggested by Lauffer.<sup>4</sup> In order to take care of the various magnification factors involved in the method, the equation given by

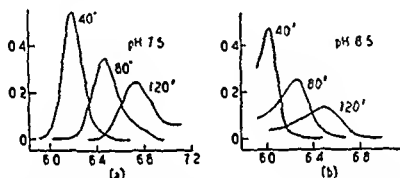


FIG. 1. Sedimentation diagrams of insulin. The abscissa scale represents the distance in cm. from the center of rotation; the ordinate scale represents the scale line displacements in mm. with a scale distance in the ultracentrifuge of 60 mm. The numbers near the peaks of the diagrams indicate lengths of time after the centrifuge had attained full speed. (a)  $s_{20} = 3.58 \times 10^{-13}$  cm. per second per unit centrifugal field; (b)  $s_{20} = 3.16 \times 10^{-13}$  cm. per second per unit centrifugal field.

Lamm (6) for the calculation of the diffusion constant by the maximum height-area method, namely

$$Dl = \frac{(\Phi)^2}{(X_m)^2} \cdot \frac{1}{4\pi}$$

was converted to the form

$$Dl = \frac{(\Phi')^2}{(X'_m)^2} \cdot \frac{1}{4\pi} \cdot \frac{1}{m^2 G^2}$$

where  $\Phi'$  and  $X'_m$  are, respectively, the areas and maximum heights measured on tracings of the scanning diagrams,  $m$  is the enlargement factor for the tracings, which was 4 in the present work, and  $G$  is the photographic enlargement factor for abscissa magnification resulting from the optical system of the diffusion apparatus. Similarly, for the method of calculation

<sup>4</sup> Lauffer, M. A., private communication.

involving the measurement of the inflection points of the diffusion curves, the equation

$$Dt = \frac{x_1^2}{2}$$

was converted to the form

$$Dt = \frac{(x'_1)^2}{2} \cdot \frac{1}{m^2 G^2}$$

where  $x'_1$  represents the abscissas for the inflection points, as measured on the tracings of the scanning diagrams. The heights on the curves for

TABLE I

*Diffusion Measurements on Crystalline Insulin*

$D_1$  represents the diffusion constants calculated by the method of "successive analysis;"  $D_2$ , the diffusion constants calculated by the "maximum height-area" method;  $D_3$ , the diffusion constants calculated from the inflection points. All values are corrected to 20° with water as the solvent.

Apparatus	Experiment No.	Protein concentration	$D_1$	$D_2$	$D_3$
		<i>per cent</i>	$10^{-1}$	$10^{-1}$	$10^{-1}$
Lamm	1	0.5	7.32		
	2	1	7.01		
	3	1	7.91		
	4	1	8.17		
Neurath-Longsworth	5	0.5		7.33	7.72
	6	0.7		6.83	7.35
	7	0.7		7.57	7.51
	8	0.7		7.96	7.68
Average $D_{20}$			7.60	7.42	7.57
Final average $D_{20}$					7.53

locating the inflection points were obtained by dividing the maximum height by  $\sqrt{e}$  (6).

The data for the measurements carried out on the insulin are presented in Table I. All values have been corrected to 20° with water as the solvent. Good agreement is shown between the results obtained at 20° with the Lamm apparatus and those obtained at 0° with the Neurath-Longsworth equipment. In addition, the average values obtained by the different methods of calculation corresponded closely to one another. Evidence for homogeneity of the insulin was provided by the excellent agreement, as shown in Fig. 2, between the experimentally determined diffusion curves and those calculated from the theory. It should be pointed out, however, that diffusion methods possess a limited sensitivity with respect to tests

for homogeneity. No significant drift in diffusion constant was observed with change in concentration of the protein. In certain cases, however, there did appear to be an appreciable variation in the values obtained from run to run. We have not yet been able to ascribe this variation to any single factor. Nevertheless, the agreement obtained in the average values and the close correspondence between the experimental and theoretical diffusion curves lend reliability to the final values.

*Molecular Weight of Insulin*—The partial specific volume,  $V$ , of insulin was found by Sjögren and Svedberg (1) to be 0.749. This value, together with the above values determined for  $s_{20}$  and  $D_{20}$ , was used in the calculation of the molecular weight of the protein by means of the equation  $M = RTs/D(1 - V\rho)$ , in which  $M$  represents the molecular weight, and  $\rho$ , the density of water at 20°. A figure of 46,000 was thereby obtained. The ratio of the observed molecular frictional constant to that of a spherical

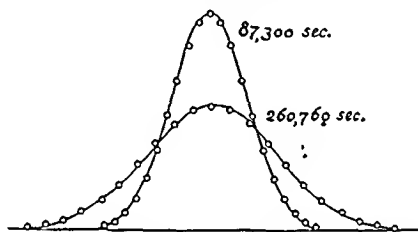


FIG. 2. Concentration gradient curves for diffusion of insulin (Experiment 5). The experimental curves are represented by continuous lines; the theoretical curves, by circles. The numbers near the peaks of the diagrams indicate the lengths of time after the diffusion was started.

molecule of the same weight was 1.18. It is interesting to note that these values describing the size and shape of insulin correspond closely to the revised values found for egg albumin (2, 9). On the basis of the present figure for the molecular weight of insulin, estimations which have been reported (10, 11) for the numbers of units of zinc, sulfur, amide nitrogen, or amino acids in the protein would have to be corrected by a factor of almost exactly 4/3.

#### SUMMARY

The sedimentation and diffusion constants of insulin have been determined on samples of carefully recrystallized and purified protein. A figure of  $s_{20} = 3.55 \times 10^{-13}$  cm. per second per unit centrifugal field obtained for the sedimentation constant confirmed the value reported by previous investigators. Diffusion measurements yielded an average value of  $7.53 \times 10^{-7}$  sq. cm. per second, which was somewhat lower than that reported

before. The agreement between the average values of the diffusion constant as obtained by each of several different methods of calculation, together with the close correspondence between the experimental and theoretical diffusion curves, was taken as evidence of the homogeneity of the material under investigation and the reliability of the final value obtained. The molecular weight of insulin estimated from the present data was found to be 46,000.

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# AN ULTRACENTRIFUGE STUDY OF REDUCED INSULIN

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The importance of the intact, oxidized state of the sulfur linkages of insulin to the physiological activity of the hormone was first clearly demonstrated by du Vigneaud and coworkers (1). In this investigation, the insulin was converted into the sulfhydryl form by treatment in neutral solution with an excess of cysteine or glutathione. It was found that the reduction invariably led to inactivation, but that subsequent oxidation was not followed by reactivation. Wintersteiner (2), in an extension of this work, found that the inactivation of insulin was complete when about one-third of the total sulfur was reduced. The rate of change of the two processes indicated, however, that actually the reduction of a very small proportion of the disulfide groups in the insulin molecule was sufficient to cause extensive inactivation. Freudenberg and Wegmann (3) have reported more recently that reduced insulin may be partially reactivated by treatment with oxygen or hydrogen peroxide in the presence of an excess of cysteine or glutathione. This was explained on the hypothesis that the insulin molecule consists of a polypeptide chain along which small branches are bridged by way of disulfide linkages, and that reactivation of the reduced insulin in the presence of high concentrations of cysteine or glutathione was possible because of the substitution of these units for the original branch chains. Stern and White (4) found that under certain conditions of reduction an insulin preparation might be obtained which contained around 5 per cent of its total sulfur in the reduced form and which possessed 50 per cent of the biological potency of the untreated protein.

In the present study we wish to obtain more definite evidence as to whether the insulin molecule actually is broken into smaller fragments when the disulfide linkages of the protein are cleaved by reduction. If the insulin molecule contains twenty-four disulfide linkages, as may be calculated from the cystine content (5) and the molecular weight (6), and these linkages are spaced at equal intervals in a polypeptide chain structure, one would expect complete reduction to produce fragments possessing molecular weights of around 2000. If the insulin molecule consists of a bundle of parallel polypeptide chains, any two adjacent ones of which are

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held together by two or more disulfide bridges, complete reduction of the molecule could yield units larger than in the first case. Should the insulin molecule possess the structure indicated by Freudenberg and Wegmann, reduction should yield a mixture of small fragments together with a larger unit of molecular weight of perhaps 30,000 to 40,000. It is also conceivable that the disulfide linkages are of no direct importance in holding parts of the insulin molecule together and that reduction of the protein would cause no decrease whatsoever in molecular weight, although in this case a change of shape might be anticipated.

It was felt that information on the question of fragmentation of insulin by means of reduction might be obtained with the aid of the ultracentrifuge. Some preliminary studies on reduced insulin in the ultracentrifuge have been made by White and Stern (7) in collaboration with Svedberg and Eriksson-Quensel. In general, the reduction appeared to have caused only rather slight changes in the behavior of the insulin, but it may be recalled that these insulin preparations were reduced to a relatively small extent. Of interest also in this connection is the study of Gralén and Svedberg (8) on another sulfur-containing protein, namely the snake poison, crotoxin. As a part of this investigation, a sample of the crotoxin was reduced with cysteine and then subjected to ultracentrifugation. It was reported that reduction had caused the formation of particles of undeterminable size, having a low molecular weight.

For the experiments on reduced insulin, we chose to vary the extent of reduction by employing different amounts of reducing agent and allowing a period of time for equilibrium to be reached. Thus, for the centrifuge runs, for example, solutions of the insulin were made up to contain 1 per cent protein in 0.2 M sodium chloride and the desired molarity of thioglycolate, the final pH was adjusted to 7.0 to 7.5, and the mixtures were allowed to stand for 24 hours at room temperature before the centrifuge runs were carried out. The 0.2 M electrolyte was introduced for the purpose of depressing charge effects which otherwise might interfere with free sedimentation.

In order to render the investigations as quantitative as possible, it was important to know the exact state of reduction of each sample to be studied. For the determinations of the amounts of cystine and cysteine in the reduced insulin, samples of the treated protein were isolated by precipitation with acid acetone, dried in a desiccator, and hydrolyzed with 20 per cent hydrochloric acid in a sealed tube in the absence of oxygen. The hydrolysates after dilution were analyzed for cysteine by iodate titration and for both cysteine and cystine by the use of the Folin phosphotungstate reagent with and without sulfite. The relationship between concentration of reducing agent and extent of reduction of the insulin is shown in Fig. 1.

A great apparent obstacle to the studies which we wished to carry out lay in the fact that, depending on the extent of reduction, the insulin was transformed to a smaller or larger extent into an acid-insoluble derivative. Thus, the insulin preparation of greatest interest for study, namely the one in which the sulfur linkages were almost completely reduced, was very largely insoluble, even at pH 7. The reduced insulin was found to be much more soluble in alkaline solution, but it was not possible to take advantage of this fact, since native insulin itself is unstable in solutions more alkaline than pH 7.5 (6). Since it was desired in the present study to avoid the use of a special dispersing agent for the reduced insulin, measurements were simply carried out on the soluble fractions.

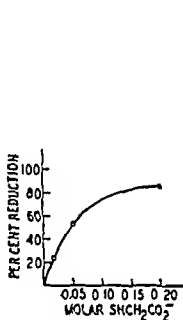


FIG. 1

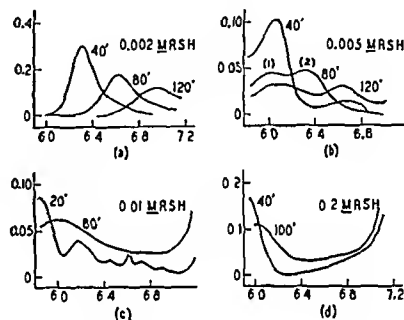


FIG. 2

FIG. 1. Effect of concentration of thioglycolate on extent of reduction of insulin in a 1 per cent solution of the protein at pH 7.

FIG. 2. Sedimentation diagrams of insulin in different concentrations of thioglycolate (RSH). The abscissas represent distances in cm. from the center of rotation, and the ordinates, scale line displacements in mm. with a scale distance in the ultracentrifuge of 60 mm. The numbers near the peaks of the diagrams indicate time in minutes after the centrifuge had attained full speed. (a)  $s_{20} = 3.96 \times 10^{-13}$  cm. per second per unit centrifugal field; (b) (1)  $s_{20} = 1.20 \times 10^{-13}$  cm. per second per unit field, (2)  $s_{20} = 3.77 \times 10^{-13}$  cm. per second per unit field; (c) and (d) sedimentation constants not determinable.

In the first experiments, insulin was treated with 0.002 M thioglycolate. Under the conditions used, the extent of reduction of the insulin corresponded to one-half disulfide linkage per molecule of protein. The reduction mixture before centrifugation was clear. From the sedimentation diagrams shown in Fig. 2, *a*, it may be seen that the homogeneity of the reduced insulin was decreased as compared with that of the untreated insulin, the sedimentation diagrams for which have been presented in a previous publication (6). There has been an apparent increase in the value for the sedimentation constant of the protein, but, as indicated by the direction of the skewness of the curves, this effect was due to the presence of a certain amount of molecules or aggregates which sedimented more



rapidly than the original insulin. The areas under the curves, which are proportional to the concentration of sedimenting substance, accounted for 80 per cent of the starting material. The loss was due, in part at least, to the presence of rapidly sedimenting soluble aggregates which went to the bottom of the centrifuge cell at the beginning of the centrifugation. No evidence of fragmentation of the insulin by reduction under these conditions was detected.

When the insulin was treated with 0.005 M thioglycolate, approximately one disulfide linkage per molecule of protein was reduced. The sedimentation curves obtained for this material revealed two distinct major boundaries and a number of minor ones. These are shown in Fig. 2, *b*. The boundary possessing a sedimentation constant,  $s_{20} = 3.77 \times 10^{-13}$ , may be considered to be unchanged or only slightly changed insulin. The more slowly sedimenting component represents either fragments of the original insulin molecule, or possibly molecules which have undergone considerable change in shape. The solution of the reduced protein before centrifugation was definitely opalescent, indicating the incipient formation of insoluble aggregates. It should be pointed out that in the curves of Fig. 2, *b* the ordinate scale was drawn 4 times that in previous diagrams. Thus, the actual concentration of unaggregated material had been greatly reduced. Because of the uncertainty of the position of the base-lines, it was not possible to make accurate calculations of the concentrations from the areas under the curves. It was very probable that again the apparent loss of sedimenting material was accountable as rapidly sedimenting aggregates.

In Fig. 2, *c* and *d*, are shown the sedimentation pictures which were obtained when the insulin was treated with 0.01 M and 0.2 M thioglycolate, respectively. In the former case, the reduction mixture was strongly opalescent and, in the latter case, approximately three-fourths of the insulin had precipitated from solution. The extents of reduction corresponded to the cleavage of three and of twenty disulfide groups, respectively. The ultracentrifugation was characterized in both cases by the rapid sedimentation of soluble protein aggregates, by the complete disappearance of the original insulin, and by the appearance of increasing amounts of low molecular weight fragments.

The final results of the insulin experiments, as summarized in Table I, do not lend themselves to as quantitative an analysis with respect to extent of fragmentation as might be hoped for. However, they do reveal some of the actual changes which occur in an insulin solution during reduction and indicate the physical changes which accompany changes in biological activity. The homogeneity of the native insulin has been found to be extremely sensitive to a very slight reduction of the sulfur linkages contained in the molecule. This is in analogy to the sensitivity of the bio-

logical activity to reduction and demonstrates that, although the extent of reduction as measured by chemical methods may be very small, the concomitant changes in physical properties of the protein are relatively great. Thus, it may be inferred that the biological inactivity of reduced insulin may not be due simply to the inactivity of the sulfhydryl insulin, but rather that it is due largely to the extensive change in physical properties of the insulin which takes place during, and possibly after, incipient reduction of the molecule. As reduction proceeds, aggregation of the products appears to be the primary change which occurs, but at the same time appreciable amounts of fragments much smaller than the original insulin also are formed.

In order to determine whether other sulfur-containing proteins would behave similarly to insulin, preliminary reduction experiments were instituted with serum albumin and with egg albumin, the former protein possessing a cystine content of around 6 per cent (9),<sup>1</sup> the latter a combined

TABLE I  
*Effect of Reduction on Particle Size of Insulin*

RSH	Reduction	Small fragments	Unchanged insulin	Soluble aggregates	Insoluble aggregates
M	per cent				
0.002	3	—	+++++	+	—
0.005	6	+	+	++++	—
0.010	12	+	—	+++	++
0.200	83	+	—	+	++++

cystine plus cysteine content of 1.3 to 1.8 per cent (10, 11). Serum albumin treated with 0.2 M thioglycolate at pH 7, in marked contrast to the insulin, exhibited no visible denaturation and appeared practically unchanged in the ultracentrifuge. Analyses for cysteine, however, revealed that little if any reduction of the protein had occurred under these conditions. When the pH of the reduction mixture was raised to 8.5, heavy precipitation of protein occurred and it was concluded that the denaturation was due to preliminary reduction. Although the study of this protein has not yet been carried further, it appears that under appropriate conditions its behavior is analogous to that of insulin. Egg albumin after treatment with 0.2 M thioglycolate even at pH 8 showed practically no change in the ultracentrifuge. From direct analyses for cysteine and cystine, it was found that the cystine of the native egg albumin was around 40 per cent in the reduced form, whereas the cystine in the reduced protein was nearly 80 per cent reduced. One may conclude, therefore, that in the

<sup>1</sup> Miller, G. L., and du Vigneaud, V., unpublished results.

case of egg albumin the reduction was not accompanied by appreciable denaturation or fragmentation.

The similarity in behavior of the high cystine-containing proteins, namely insulin and serum albumin, as contrasted with the low cystine-containing protein, egg albumin, suggests that the denaturation of the former was incumbent on preliminary reduction. The negative result with the egg albumin indicated further that the denaturation of insulin and serum albumin was made possible by the reducing action of the thioglycolate rather than by any general denaturing property of the reagent.

#### EXPERIMENTAL

*Method of Reduction*—The thioglycolic acid used was purified by fractional distillation *in vacuo*. A typical reduction experiment was carried out as follows: 10.75 mg. of crystalline insulin were dissolved in 0.40 cc. of 0.02 N hydrochloric acid added at the rate of 0.1 cc. at a time with stirring. 0.25 cc. of alkaline reducing agent made up of 1 part of 0.04 M lithium thioglycolate and 4 parts of 0.05 M lithium hydroxide was then added. Finally 0.07 cc. of 0.05 N lithium hydroxide and 0.20 cc. of water were added to give a solution of 1 per cent insulin in 0.002 M thioglycolate at pH 7.0. The mixture was allowed to stand at 20° for 24 hours, at the end of which time 0.1 cc. of 2.0 M sodium chloride was added and the ultracentrifuge measurement carried out.

For the isolation of the reduced insulin preparatory to analysis for cystine and cysteine content, the reductions were carried out as outlined above with the exceptions that 40 mg. samples of insulin in 4 cc. of reducing mixture were used and the addition of the sodium chloride was omitted. The reduced proteins were isolated by adding the reduction mixtures to 40 cc. of acetone containing 1 cc. of 1 N hydrochloric acid. The precipitates which separated were allowed to settle, the supernatant liquids decanted, and the remaining suspensions centrifuged at 3000 R.P.M. The precipitates were washed three times at the centrifuge with 3 cc. portions of acetone, and were then dried in a vacuum desiccator. In the experiments with egg albumin, 200 mg. samples of protein in 4 cc. of solution were used. The reduced egg albumin preparations were isolated as described above except that 40 cc. of acetone containing 2 cc. of concentrated hydrochloric acid were used. Under these conditions the final product was a fine powder which was more easily handled than the isolated insulin preparations. The lithium salt of the thioglycolic acid was employed in preference to the sodium salt because of the greater solubility of the former in the acid acetone employed in the isolation procedure.

*Methods of Analysis*—The first step in the procedure consisted in the hydrolysis of the sample of protein with a measured amount of a hydro-

lyzing mixture containing 4 parts of concentrated hydrochloric acid, 1 part of glacial acetic acid, and 3 parts of water. The hydrolyses were carried out in sealed tubes and in an atmosphere of nitrogen. A bath temperature of 115° was maintained and the heating continued for 24 hours. Acetic acid was employed in place of formic acid (5) because of decomposition of the latter on heating in sealed tubes and because the acetic acid aided in dissolving the proteins at the beginning of the digestion. The final hydrolysates were then diluted to 1 N hydrochloric acid. Suitable aliquots were treated with 5 N potassium iodide to give a concentration of 1 N iodide, and the solutions were then titrated with 0.01 N potassium biiodate. In this way a measure of the amounts of cysteine present was obtained.

Suitable aliquots were also analyzed for cystine and cysteine by the following modification of the Folin method. To 1 cc. of diluted hydrolysate containing 0.1 to 0.4 mg. of cystine plus cysteine in 1.0 N hydrochloric acid was added 1 cc. of 0.4 M sodium sulfite followed by 4 cc. of 0.4 M disodium acid phosphate and 1 cc. of phosphotungstate color reagent prepared according to Folin's 1934 directions (12). After 5 minutes the colors were measured in a Pulfrich photometer in terms of extinction coefficient. For determinations of cysteine alone, the 0.4 M sodium sulfite was substituted by an equal volume of 0.4 M disodium acid phosphate. Color intensities given by standard samples of cystine or cysteine were easily reproduced; so that a calibration curve could be employed. In the presence of sulfite, cysteine gave twice the color of an equal weight of cystine and twice the color of the same sample of cysteine in the absence of sulfite. This relationship is required by the theory of the reactions involved (13). The extinction coefficient due to cystine when present in a mixture containing cysteine was calculated by the expression  $(C_{\text{SO}_3} - 2C_0)/2$ , where  $C_{\text{SO}_3}$  is the extinction coefficient of the unknown determined in the presence of sulfite and  $C_0$  is the extinction coefficient measured in the absence of sulfite. Ten different samples of cysteine, varying in amount from 2 to 7 mg., were carried through the hydrolysis procedure employed with the proteins. An average loss of  $0.18 \pm 0.05$  mg. of cysteine resulted. This loss was found to be due to oxidation to cystine. A correction of this amount was therefore applied to all analyses.

The determinations of cystine and cysteine in the insulin and egg albumin were carried out as follows: In one experiment, 5 mg. of crystalline insulin were digested with 0.83 cc. of hydrolyzing mixture. The hydrolysate was diluted to 5 cc. and 1 cc. aliquots were analyzed by the Folin method. There was no cysteine present. The cystine content of the insulin was 11.3 per cent, uncorrected for ash and moisture. In another experiment, 32.7 mg. of insulin which had been reduced in 0.05 M thioglycolate were digested with 1.66 cc. of hydrolyzing mixture and the final hydrolysate was

diluted to 10 cc. To a 5 cc. aliquot, 1 cc. of 5 N potassium iodide was added and the mixture was titrated with 0.01 N biiodate. According to this titration, the reduced insulin under analysis contained 4.66 per cent cysteine. A 1 cc. aliquot of the diluted hydrolysate gave with the Folin method in the absence of sulfite an extinction coefficient indicative of 5.25 per cent cystine. It was assumed that the difference was due to non-cysteine reducing substances and that these extraneous substances would not give enhanced color in the presence of sulfite. A determination was then made on a 1 cc. aliquot in the presence of sulfite. The color obtained under these conditions, when corrected for that due to the cysteine present as determined by iodine titration and for that due to the extraneous reducing substances, indicated a cystine content of 5.87 per cent. When corrected for the conversion of 0.18 mg. of cysteine to cystine under the conditions of the hydrolysis, the per cent cysteine became 5.21, the per cent cystine, 5.32. The extent of reduction was therefore 49 per cent. For the egg albumin, 200 mg. samples of the recrystallized protein gave by the biiodate titration method 0.48 per cent cysteine. When corrected for conversion of cysteine to cystine, the figure became 0.58 per cent. The reduced egg albumin, after isolation, contained 1.07 per cent cysteine. Determinations by the Folin method revealed the presence in the hydrolysate of extraneous substances which reduced the phosphotungstate reagent in the absence of sulfite. When corrections were applied as in the insulin analyses, an average value of 1.38 per cent cystine plus cysteine was obtained.

*Ultracentrifuge Determinations*—Studies on the sedimentation of the reduced insulin were made with the oil-driven ultracentrifuge at a speed of 65,000 R.P.M. Observations of the sedimenting boundaries were made by the Lamm scale method (14).

For the calculation of the theoretical areas under the refractive index curves for the sedimentation boundaries, determinations of the refractive index increment of insulin were first carried out. These measurements were obtained with the aid of a differential prism apparatus employed in the Upsala laboratory. The refractive index increment of the insulin was found to be  $1.88 \times 10^{-3}$  for a wave-length of 436 m $\mu$  and  $1.82 \times 10^{-3}$  for a wave-length of 546 m $\mu$ . These values each represent the average of two determinations.

#### SUMMARY

Preparations of insulin in which the disulfide linkages were reduced with thioglycolate at pH 7 to 7.5 have been studied in the ultracentrifuge. The primary change in properties of the insulin on reduction was an aggregation of the reduced molecules to form particles of much greater size than the

original protein. In addition, the formation of small amounts of low molecular weight fractions was demonstrable.

The well known high degree of sensitivity of the biological activity of insulin to reduction of the hormone was found to be paralleled by a rapid change in physical properties of the protein. It was concluded therefore that the inactivity of reduced insulin may be the result of its denatured and aggregated state rather than simply its sulfhydryl structure.

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# ULTRACENTRIFUGE AND DIFFUSION STUDIES ON NATIVE AND REDUCED INSULIN IN DUPONOL SOLUTION

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In a study of reduced insulin by means of the ultracentrifuge (1), it was reported that a certain amount of fragmentation of the insulin occurs during reduction, but that the major portion of the reduced protein becomes aggregated. In order to gain more information concerning the actual size of the units which make up the aggregated material, experiments have been instituted in which special agents were employed for dispersing the protein. Sodium salicylate as a dispersing agent was found to have comparatively little effect. Urea, in concentrations of 25 per cent or greater, maintained a clear solution of the reduced insulin for a day or two. Commercial Duponol,<sup>1</sup> on the other hand, in amounts as low as 2 per cent, was found to maintain clear solutions of the reduced insulin almost indefinitely. Because of certain difficulties which may be encountered in ultracentrifuge experiments when high concentrations of urea are employed, the Duponol was chosen as the more suitable dispersing agent for the present study.

The principal organic components of the commercial Duponol were the sodium salts of sulfated aliphatic alcohols of chain length  $C_8$  to  $C_{18}$ . The preparation contained in addition about 50 per cent of inert material consisting mainly of sodium sulfate with a small amount of sodium chloride. Before the Duponol was used in the present investigations, it was subjected to a partial purification by a single reprecipitation from water. Elementary analyses carried out on the partially purified material revealed that the detergent still contained about 15 to 20 per cent of impurity in the form of sodium sulfate. From the studies of Hartley and Runnicles on paraffin chain detergents (2), it was to be expected that the Duponol would exist in solution in micellar form. In order to determine the size and shape of the Duponol micelles, measurements were made of the sedimentation and diffusion constants of the material. The measurements were carried out in a medium of 0.2 M lithium chloride which, on the basis of the data of Hartley and Runnicles, provided sufficient electrolyte to suppress interfering charge

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<sup>1</sup> Duponol (WA flakes), manufactured by E. I. du Pont de Nemours and Company, Inc.



effects. From a series of determinations, average values of  $s_{20} = 0.71 \times 10^{-13}$  cm. per second per unit centrifugal field and  $D_{20} = 10.5$  sq. cm. per second were obtained. It was found in addition that the rates of sedimentation and of diffusion did not change significantly when the concentration of Duponol was varied from 0.2 to 2.0 per cent. A value of  $V = 0.87$  was obtained for the partial specific volume of the detergent by measurements carried out on a sample of analytically pure sodium dodecyl sulfate<sup>2</sup> more recently studied. When the values  $s_{20} = 0.71 \times 10^{-13}$ ,  $D_{20} = 10.5 \times 10^{-7}$ , and  $V = 0.87$  were substituted in the formula  $M = RTs/D(1 - V\rho)$ , a micellar weight of 12,500 was obtained. The frictional ratio,  $f/f_0$ , was equal to 1.24, which indicated a degree of symmetry comparable to that of the native insulin (3).

In qualitative experiments with mixtures of unreduced insulin and Duponol, it was observed that the native insulin could be precipitated from solution by very small amounts of Duponol, but that the precipitated material could subsequently be redissolved by means of additional amounts of the detergent. On the basis of Anson's findings that detergents have a strong denaturing effect on proteins (4), it seemed likely that the small amount of Duponol caused a denaturation of the insulin, whereas the higher concentrations of the detergent then exerted a dispersing effect on the denatured protein. In order to minimize the effect of the Duponol in causing any aggregation and precipitation of the insulin, it appeared desirable to employ a considerable excess of the detergent. Qualitatively, a concentration of 2 per cent Duponol for 1 per cent insulin appeared adequate for this purpose. The first sedimentation and diffusion experiments to be considered, therefore, are those carried out under these conditions. An electrolyte concentration of 0.2 M lithium chloride was maintained in order to suppress undesirable charge effects. The sedimentation diagrams presented in Fig. 1 show the results obtained with a solution of 1 per cent unreduced insulin in a medium of 2.0 per cent Duponol. It may be seen that a single, sharp boundary was obtained. A measurement of the areas under the curves showed that both the insulin and the Duponol were present in the same boundary. It appeared, therefore, that a new substance, an insulin-Duponol complex, had been formed. The sedimentation constant,  $s_{20} = 1.61 \times 10^{-13}$ , was intermediate between that of Duponol alone and that of pure insulin (3). In a diffusion experiment with 1 per cent insulin in 2 per cent Duponol, quite good diffusion curves were obtained and a diffusion constant of  $8.98 \times 10^{-7}$  was calculated. On the assumption that the insulin-Duponol complex would possess a mean partial specific volume,  $V = 0.83$ , a micellar weight of 27,600 was estimated. Since this value was

<sup>2</sup> A sample of analytically pure sodium dodecyl sulfate was generously put at our disposal by Dr. S. Lenher of E. I. du Pont de Nemours and Company, Inc.

lower than the molecular weight of insulin alone, it was concluded that the Duponol had caused a dissociation of the native insulin. Furthermore, since the insulin and Duponol appeared to exist solely as a complex, the molecular weight of the insulin portion of the complex could be calculated to be approximately one-third of the value of 27,600. The frictional ratio,  $f/f_0$ , was found to be 1.20, indicating that the complex possessed essentially the same shape as that of each of its components when present alone.

In order to determine the effect of reduction of the protein, ultracentrifuge experiments were next carried out in 2 per cent Duponol containing concentrations of lithium thioglycolate of 0.005, 0.02, and 0.2 M. According to previous work (1), the 0.2 M thioglycolate was sufficient to produce quite complete cleavage of disulfide linkages in the protein. In

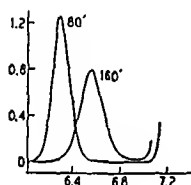


FIG. 1

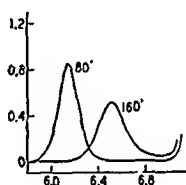


FIG. 2

FIG. 1. Sedimentation diagrams of a mixture of 1 per cent insulin and 2 per cent Duponol. The abscissas represent distances in cm. from the center of rotation. The ordinates indicate scale line displacements in mm. with a scale distance in the centrifuge of 60 mm. The numbers near the peaks of the diagrams indicate the time in minutes after the centrifuge had attained full speed.  $s_{20} = 1.61 \times 10^{-13}$  cm. per second per unit centrifugal field.

FIG. 2. Sedimentation diagrams of a mixture of 1 per cent insulin and 1 per cent Duponol.  $s_{20} = 2.20 \times 10^{-13}$  cm. per second per unit centrifugal field.

special experiments, it was found that the presence of Duponol did not interfere with the reduction of disulfide linkages in the insulin. The sedimentation constants obtained in the several runs were  $1.62 \times 10^{-13}$ ,  $1.49 \times 10^{-13}$ , and  $1.45 \times 10^{-13}$ , respectively. The sedimentation diagrams which were obtained in these experiments were very similar to those obtained with the unreduced insulin, as shown in Fig. 1. Furthermore, from an inspection of the extent of spreading of the sedimentation curves, it was expected that the rates of diffusion in these cases would differ very little from that of the corresponding mixture of native insulin and Duponol described above. It was concluded, therefore, that the state of reduction of the insulin had rather little effect on the size of the micellar units of the insulin-Duponol complex when 2 per cent Duponol was employed. It was quite probable, however, that the apparent dissociating effect of the deter-

gent on the protein molecule had in some way obscured any effects which might have been due to reduction alone.

In order to obtain a clearer insight into the changes which took place in mixtures of Duponol with the native or the reduced insulin, further studies were carried out in which diminishing proportions of the dispersing agent were employed. When the concentration of dispersing agent was reduced to 1.0 per cent, single, symmetrical boundaries again were obtained, as exemplified by the sedimentation diagrams shown in Fig. 2. The rates of sedimentation were higher, however, than in the runs carried out with the insulin in a medium of 2 per cent Duponol. This was presumably due to the existence of larger micelles in the present case. Thus, the complex of unreduced insulin with Duponol sedimented at a rate of  $s_{20} = 2.20 \times 10^{-13}$ , whereas the insulin preparations reduced with 0.005, 0.02, and 0.2 M thioglycolate yielded sedimentation constants of  $2.34 \times 10^{-13}$ ,  $2.18 \times 10^{-13}$ , and  $2.04 \times 10^{-13}$ , respectively.

When the ultracentrifuge measurements were carried out with insulin in 0.5 per cent Duponol, still higher rates of sedimentation were observed. In distinction to previous results, however, the sedimenting boundaries obtained under these conditions showed a more definite change in sedimentation rate with variation in extent of reduction. For example, the untreated insulin in the presence of 0.5 per cent Duponol gave a sedimentation constant of  $3.51 \times 10^{-13}$ , while the insulin preparations reduced with 0.005, 0.02, and 0.2 M thioglycolate yielded the significantly lower sedimentation rates of  $2.74 \times 10^{-13}$ ,  $2.57 \times 10^{-13}$ , and  $2.42 \times 10^{-13}$ , respectively. Furthermore, in certain cases, the sedimenting boundaries were no longer symmetrical as in the preceding studies. In the particular experiments with untreated insulin and with insulin reduced with 0.005 M thioglycolate, it was possible to resolve the sedimentation curves into two components. This is shown by the diagrams of these boundaries presented in Fig. 3, *a* and *b*. For the construction of the resolved curves, it was assumed that the two components consisted of one fraction possessing a sedimentation rate of about  $2.7 \times 10^{-13}$ , and a second fraction with a sedimentation rate of  $3.6 \times 10^{-13}$ . Because of the fact that there was approximately twice as much insulin as Duponol present in the original mixtures, the component shown in either of the sedimentation diagrams to be present to the greatest extent could be identified as being primarily protein in nature. In the case of the unreduced protein in the presence of Duponol, as shown in Fig. 3, *a*, it may be seen that the component present in greatest amount also was the most rapidly sedimenting one. With increasing extent of reduction, however, the rapidly sedimenting component decreased in amount and gradually merged with the more slowly sedimenting component. This is shown by the character of the sedimentation curves presented in Fig. 3, *a*, *b*, and *c*,

for the reduced insulin. Since the more slowly sedimenting component possessed in each case a sedimentation rate higher than that of Duponol when present alone, it represented a complex of the Duponol with breakdown units of the protein. The effects of reduction just described might be explained on the basis that the cleavage of disulfide bonds had caused a fragmentation of the insulin molecule. However, because of the fact demonstrated in earlier experiments, that higher concentrations of Duponol effectively dispersed the unreduced insulin and the reduced insulin into

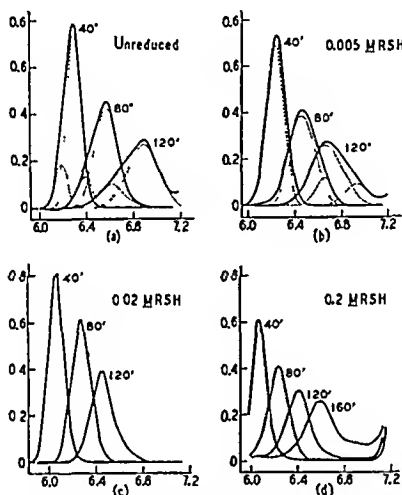


FIG. 3. Sedimentation diagrams of a mixture of 1 per cent insulin and 0.5 per cent Duponol at different stages of reduction of the insulin. (a)  $s_{20} = 3.51 \times 10^{-13}$ ; (b)  $2.74 \times 10^{-13}$ ; (c)  $2.57 \times 10^{-13}$ ; (d)  $2.42 \times 10^{-13}$  cm. per second per unit centrifugal field. The curves shown with broken lines represent the resolution of the continuous curves into two component curves. The one component curve represents a substance with  $s_{20} = 2.7 \times 10^{-13}$ ; the other component curve represents a substance with  $s_{20} = 3.6 \times 10^{-13}$ .

subunits of about the same size, it is quite probable that the breakdown of the insulin by reduction which was apparent in the present experiments with low concentrations of detergent was in reality due indirectly to the action of the detergent alone. If the detergent possessed a special affinity for the reduced protein, it might be expected to exert a greater dispersive action on this material than on the untreated protein. For reasons which will be discussed later, this interpretation appears to be a reasonable one.

In further experiments, the insulin was reduced with 0.005 M thioglycolate in 0.2 per cent Duponol and a control run again made with untreated in-

sulin. Detailed experiments with higher concentrations of the thioglycolate were not carried out, since under these conditions the amount of Duponol used, namely 0.2 per cent, was insufficient to keep the reduced insulin completely in solution. The sedimentation diagrams in the ultracentrifuge experiments which were made are shown in Fig. 4. Abnormally high sedimentation rates were observed both with the untreated and with the reduced insulin. In the former case the sedimentation constant was  $4.20 \times 10^{-13}$ , in the latter case,  $4.00 \times 10^{-13}$ . These findings, though unexpected, actually were in accord with the results of previous experiments, in so far as the micellar size of the insulin-Duponol complex seemed to increase with decreasing proportions of the detergent. This phenomenon may be explained on the hypothesis indicated earlier; namely, that small amounts of Duponol cause a denaturation and aggregation of the protein, whereas higher concentrations of the detergent effect a subsequent dispersion and re-solution of the aggregated particles. In the cases of the

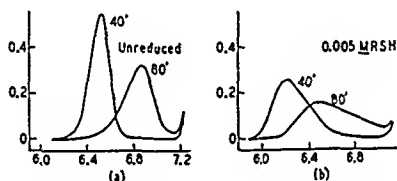


FIG. 4. Sedimentation diagrams of a mixture of 1 per cent insulin and 0.2 per cent Duponol at different stages of reduction of the insulin. (a)  $s_{20} = 4.20 \times 10^{-13}$ ; (b)  $4.00 \times 10^{-13}$  cm. per second per unit centrifugal field.

abnormally high sedimentation rates just described, it may be assumed that the amount of detergent present was adequate only for dispersing the denatured, aggregated protein into units of a size which was indicated roughly by the magnitude of the sedimentation constants. For the particular case of the reduced insulin, as shown in Fig. 4, b, the character of the skewness of the sedimentation curves suggests that the reduction of disulfide linkages in the insulin served as an additional denaturing and aggregating influence upon the protein and that the limited amount of Duponol present was even less adequate for dispersing the reduced protein than the unreduced protein.

In order to determine the effect of Duponol on the sedimentation behavior of other proteins, preliminary experiments were carried out with horse serum albumin. Native horse serum albumin has been found to possess an  $s_{20}$  of  $4.46 \times 10^{-13}$  (5). A mixture of 1 per cent serum albumin<sup>1</sup> in 0.5 per cent Duponol yielded on ultracentrifugation a single, fairly sym-

<sup>1</sup> We wish to thank Dr. Harry Svensson for putting a sample of purified horse serum albumin at our disposal.

metrical boundary with a sedimentation constant of  $3.76 \times 10^{-13}$ . The areas under the curves indicated the presence of the Duponol as well as the serum albumin. It appears, therefore, that the behavior exhibited towards Duponol by insulin may be common to other proteins. In this connection, the results of Sreenivasaya and Pirie (6) and of Smith and Pickels (7) also should be mentioned. The former investigators reported that the detergent, sodium dodecyl sulfate, caused the breakdown of tobacco mosaic virus protein into fragments which were too small to be sedimented in the ultracentrifuge under a force of 17,000 times gravity. Smith and Pickels, in ultracentrifuge studies on the action of sodium dodecyl sulfate on the chlorophyll-protein complex obtained from spinach, reported a similar breakdown of protein and noted in addition that the detergent sedimented along with the fragments of the protein.

#### DISCUSSION

It is of interest to attempt to interpret the sedimentation and diffusion data in terms of the mechanism of the action of detergents in denaturing proteins and in dispersing the denatured proteins. Hartley (8) has explained the dispersion of non-polar substances in aqueous solutions of detergents on the basis of the dissolving of the non-polar substance in the liquid paraffin interior of the detergent micelles. The solubility of partially polar, partially non-polar substances in detergents was proposed by Lawrence (9) to be the result of a dissolving of the non-polar portions of the substance in the paraffin interior of the micelles, while the polar portions of the solute are attracted to the hydrophilic, ionized surface of the micelles. Protein molecules, such as those of the insulin used in the present studies, would seem to fall in the latter category, since they possess both polar and non-polar groupings. In particular, denatured proteins, which by nature are more hydrophobic than native proteins, might be expected to be especially affected by detergents.

From Cohn's studies of the influences of different groupings on the solubility of amino acids and proteins (10), one would conclude that the hydrophobic character acquired by proteins during denaturation results either from the loss of polar groups or from the gain in non-polar groups. Since, however, titration studies have not revealed any great differences between denatured and native proteins with respect to content of polar acid and base-combining groups (11), it seems likely that the change in properties undergone by proteins when they are denatured results primarily from the liberation or formation of non-polar groups, possibly by an opening up or unfolding of the protein molecule as suggested by Mirsky (12). Accordingly, one might conceive of the denaturation of proteins by detergents as taking place in a series of steps such as the following. The detergent

micelles may first attract and combine with the hydrophobic groupings of the protein molecule. Eventually hydrophobic groupings in more inaccessible parts of the native protein unit may be affected. The resulting forces may then cause a rupture of the protein and a severance of the normal bonds which maintain the native protein unit. The subunits thus formed may possess a preponderance of hydrophobic groupings; so that the material becomes water-insoluble, aggregates, and precipitates from solution. Larger amounts of Duponol may then cause a re-solution of the precipitate owing to the further combination of the Duponol with the hydrophobic groups of the material and the subsequent solubilizing effect of the increased proportions of attached hydrophilic sulfate radicals. As the concentration of Duponol is increased, the detergent probably disperses the aggregated material more and more completely and combines with the ultimate subunits to form relatively homogeneous complexes of detergent with the subunits.

The striking difference in the behaviors of the reduced and the native insulin under appropriate conditions of treatment with Duponol requires, however, a special explanation. As has been demonstrated in a previous investigation (1), reducing agents, as represented by thioglycolate, have a powerful effect in aggregating insulin and eventually rendering it insoluble in water. This effect was shown to be incumbent on the ability of the reducing agent to cleave disulfide linkages in the protein. From the fact that the reduced insulin becomes quite water-soluble in the presence of detergent, it must be concluded that there exists some sort of special attraction and interaction between the two substances. It appears very likely that the cleavage of disulfide linkages within the protein molecule leads to the formation of a type of altered protein which is much more non-polar than the type of altered protein which is formed by the simple denaturing action of detergent alone on the untreated protein. On this basis it would be expected that the reduced protein would have a greater affinity for the organophilic detergent and in turn might be more effectively dispersed by it. Such a mechanism would explain the finding described earlier; namely, that with appropriate concentrations of Duponol, the detergent appeared to disperse the reduced protein into smaller units than it dispersed the untreated protein. Sufficiently high concentrations of detergent, on the other hand, could conceivably by mass action effectively disperse the unreduced protein as well as the reduced protein. From the experimental results which were obtained with higher proportions of detergent, this appears to be the case.

The unusual properties of micellar detergents may in themselves cause effects on sedimentation and diffusion rates not yet realized or understood. Because of this possibility, an alternative interpretation of the results of the

above sedimentation and diffusion studies should also be considered. The experimental finding, that mixtures of protein and detergent in certain proportions yield sedimentation constants intermediate between those of each of the component substances when sedimented in the absence of the other, suggests that a simple attractive force exists between the micelles of the two individual substances in solution. This interpretation implies attractive forces operating at appreciable distances in the solvent, whereas the previous interpretation was based on the assumption that complexes were formed due to mutual solubility effects of the detergent micelles and the protein. The former type of attraction would be similar, in effect, to the attraction between the ions of sodium and proteinate in a pure solution of sodium proteinate. Thus, the small, slowly sedimenting sodium ions tend to retard the rate of sedimentation of the large protein ions. In the case of the mixture of insulin and Duponol, the more slowly sedimenting detergent micelles might be expected to retard the sedimentation of the protein molecules. Furthermore, higher proportions of the detergent would cause a greater and greater retardation effect. With regard to the diffusion data, it may be noted that the diffusion constant of the mixture of 1 per cent insulin in 2 per cent Duponol was intermediate between the values given by each component studied singly. This result also could be expected if the attraction between the detergent micelles and protein molecules caused an accelerated movement of protein and a retarded movement of detergent. In the case of sedimentation and diffusion measurements with solutions of proteins alone, these anomalous effects are effectively eliminated by the addition of an excess of electrolyte to the media. Precautions in this direction have been taken in the present investigations. It is questionable, however, whether an excess of electrolyte could prevent similar interfering effects during the sedimentation and diffusion of mixtures of detergent and protein. Therefore, until further study has established more conclusively that free sedimentation and diffusion may be expected under the conditions which have been employed, final conclusions as to the correct interpretation of results must be reserved.

#### EXPERIMENTAL

*Partial Purification of Commercial Duponol*—30 gm. of Duponol (WA flakes) were dissolved in 200 cc. of warm water. After the solution was allowed to stand at 16° for 24 hours, the first crop of precipitated material was filtered and discarded. The filtrate was then placed in the refrigerator and the second crop of material which separated was filtered, washed with cold water and acetone, and dried. 5 gm. of material were obtained.

The partially purified material contained 42.11 per cent carbon, 7.44



per cent hydrogen, 13.06 per cent sodium, and 18.62 per cent sulfur.<sup>4</sup> The partial specific volume,  $V$ , was found to be 0.76.<sup>5</sup> A sample of pure sodium dodecyl sulfate,<sup>2</sup> more recently studied, was found to have a partial specific volume of 0.87. On the assumption that the alkyl sulfate present in the purified Duponol was best represented by chain lengths of 12 carbon atoms, the values for carbon, hydrogen, sodium, and partial specific volume indicated the presence of 15 to 20 per cent impurity in the form of sodium sulfate. The high sulfur value indicated the presence of other sulfur-containing impurities of unknown nature.

The refractive index increment for the partially purified Duponol was measured in an Abbé refractometer and found to be approximately  $1.1 \times 10^{-3}$ .

*Preparation of Mixtures of Insulin and Duponol*—For the sedimentation and diffusion studies on mixtures of Duponol with native or reduced insulin, the solutions were made up according to the general procedure described in a previous paper (1), with the exception that the desired proportion of Duponol was incorporated in the mixtures. A stock solution containing 10 per cent of the partially purified detergent was employed for this purpose.

*Sedimentation Measurements*—The determinations of rates of sedimentation were carried out in an oil-driven ultracentrifuge at a speed of 65,000 R.P.M. Observations of the sedimenting boundaries were made by the Lamm scale method (13). The temperature within the rotating centrifuge cell during the various runs was around 25°. A medium of 0.2 M lithium chloride was used for all sedimentation measurements in order to minimize the interfering influence of electrical charges on the movement of the colloidal particles.

In calculations of the sedimentation rates of the mixtures of insulin and Duponol, no corrections were applied for the effects of the detergent on the specific gravity or viscosity of the medium. This procedure was adopted because of the fact that the detergent sedimented with the protein and therefore would not be expected to exert any effects for which corrections would be required. These corrections, even if applied, however, would be quite small and would not alter the results significantly.

The value for the refractive index increment given above for the Duponol, namely  $1.1 \times 10^{-3}$ , together with the appropriate value reported previously for insulin (1), was used for the estimation of the theoretical areas under the various sedimentation curves which were obtained. On the

<sup>4</sup> The elementary analyses were carried out by Dr. A. Elek, The Rockefeller Institute, New York.

<sup>5</sup> The authors wish to thank Dr. C. Drueker at the Institute of Physical Chemistry, Upsala, for making the measurements of partial specific volume.

average, the measured areas represented about 80 per cent of the calculated areas. The loss was accountable, in part at least, by the presence of non-sedimenting impurities present in the Duponol preparation.

*Diffusion Measurements*—Diffusion measurements were carried out at 20° in the metal diffusion cell and by the refractometric method as described by Lamm (13). A medium containing 0.2 M lithium chloride was employed in all experiments for the reasons indicated above. Calculations of the diffusion constants were made by the method of "successive analysis" (13).

In the particular diffusion experiments carried out with Duponol alone, it was found that there was no significant variation with different concentrations of the Duponol. At the same time, however, duplicate determinations at any given concentration often gave irregular results. Lamm and Högberg (14) have observed that in the cases of ordinary soaps it was necessary to allow the solutions to stand for some time at the desired concentration before diffusion measurements were made, and also that still better results were obtainable when a given concentration of the diffusing substance on the one side of the boundary was placed opposite a lower concentration of the same substance on the other side of the boundary. We therefore allowed our solutions to stand for 24 hours before making diffusion runs, and, by using 0.5 per cent Duponol on the one side of the boundary and 1.0 per cent on the other side, or 0.2 per cent on the one side and 0.7 per cent on the other, obtained diffusion constants of  $10.4 \times 10^{-7}$  and  $10.7 \times 10^{-7}$  sq. cm. per second, respectively. The results from these runs fell inside the extreme values obtained in the previous runs.

#### SUMMARY

Ultracentrifugation and diffusion studies have been carried out on native and reduced insulin preparations in various concentrations of the commercial detergent, Duponol. Preliminary investigations on the Duponol alone revealed that the detergent existed in aqueous solution in the form of micelles, possessing a micellar weight of around 12,500.

In studies with mixtures of 1 per cent native insulin in the presence of 2 per cent Duponol, it was found that the two substances existed in close association with one another to form a new substance, an insulin-Duponol complex. The complex possessed a micellar weight of about 27,600, which indicated that the original insulin molecule had undergone a dissociation into subunits. With preparations of reduced insulin in the presence of the same proportion of detergent, essentially the same results were obtained. It was concluded, therefore, that under the conditions of these experiments the state of reduction of the insulin had no appreciable effect on the size of the micellar units of the insulin-Duponol complex. It was pointed out,

however, that the apparent dissociating effect of the detergent on the protein may have obscured any effects which might have been due to reduction alone.

In further studies carried out with diminishing proportions of the detergent, complexes of ever increasing size were obtained. From this and certain qualitative results which were described, it was concluded that small amounts of the detergent caused a denaturation and aggregation of the insulin and that higher concentrations of the detergent caused a redispersion and dissociation of the denatured protein. The extent of dissociation of the protein thus appeared to depend directly on the proportion of detergent present. In further distinction to results obtained with 1 per cent insulin in the presence of 2 per cent Duponol, it was found that, with lower concentrations of the detergent, the reduced insulin showed a greater affinity for the detergent than did the unreduced protein, with the result that at certain concentrations of the Duponol the detergent effectively dissociated the reduced insulin into smaller units than it was able to dissociate the untreated protein. This was explained on the hypothesis that the insulin acquired during reduction of its disulfide linkages a particularly strong non-polar character; so that its affinity for the non-polar portion of the detergent was thereby enhanced. The fact that higher concentrations of detergent could effectively dissociate the reduced and native proteins into units of approximately the same size was explained on the basis of the effect of mass action of the detergent.

Because of the possibility that the unusual properties of micellar detergents might in themselves cause effects on sedimentation and diffusion rates not yet realized or understood, another interpretation of the experimental results also was presented and discussed briefly.

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# QUANTITATIVE INVESTIGATIONS OF AMINO ACIDS AND PEPTIDES

## IX. SOME PHYSICAL PROPERTIES OF *l*(-)-HISTIDINE\*

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The investigation of some physical properties of amino acids, undertaken in the authors' laboratory, has been extended in the present report to *l*(-)-histidine. The solubility and specific rotations of *l*(-)-histidine were established by methods which differ somewhat from those employed previously with *l*(-)-leucine (1). Studies were made of the variation of specific rotation with temperature, concentration of solute, and character of the solvent.

### *Preparation of Purified Natural l(-)-Histidine*

100 gm. of commercial *l*(-)-histidine monohydrochloride monohydrate<sup>1</sup> were dissolved in 125 ml. of boiling water and 85 ml. of concentrated ammonium hydroxide were added to the solution. Crystallization of the histidine began as the solution cooled. When the temperature of the solution reached 50°, 100 ml. of 96 per cent ethanol were added and the mixture was allowed to stand overnight in the refrigerator. The suspension was filtered and the crystals were washed free of chloride with 96 per cent ethanol. The histidine, recrystallized from water with the aid of ethanol and dried overnight in air, contained less than 0.05 per cent moisture determined by drying the product to constant weight in a partial vacuum at 75°.

### *Purity of Purified Natural l(-)-Histidine*

**Nitrogen Analysis**—The nitrogen values were low and inconsistent when determined by a conventional Kjeldahl method. Vickery<sup>2</sup> has shown that

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<sup>1</sup> Purchased from A. D. Mackay, New York.

<sup>2</sup> Private communication from Dr. H. B. Vickery, Connecticut Agricultural Experiment Station, New Haven, Connecticut, November, 1941.

nitrogen in histidine may be determined quantitatively by Kjeldahl analysis if a mercury catalyst and an 8 hour digestion period are employed. This information was not available at the time of the authors' experiments.

*Equivalent Weight*—The perchloric acid titration procedure of Harris (2), as modified by Toennies and Callan (3), was first used. Glacial acetic acid was purified by fractional distillation and crystallization of the commercial 99 per cent product. The fraction boiling at 116–117° was recrystallized until the freezing point was 16.5°, the density at 20° was 1.0497, and the equivalent weight was 100.4 per cent of the theoretical value. Acetic acid solutions of perchloric acid were prepared by adding to 65 per cent aqueous perchloric acid solution a quantity of redistilled acetic anhydride exactly equivalent to the water present and diluting the mixture with purified glacial acetic acid. The end-point of the perchloric acid titration of amino acids with crystal-violet indicator is unsatisfactory unless the water content of the acetic acid is less than 0.2 per cent. Under anhydrous conditions the color changes from dark green to light green and thence to yellow are sharp and reproducible with high precision.

The 0.2 N perchloric acid-acetic acid solutions were standardized against analytically pure glycine.<sup>3</sup> The precision and accuracy of the method were found to be  $100.6 \pm 0.3$  per cent in analyses of analytically pure *dl*-serine,<sup>4</sup> *dl*-phenylalanine,<sup>5</sup> and *dl*-valine.<sup>6</sup> All volumetric apparatus was calibrated for acetic acid delivery.

In the perchloric acid titration of histidine, which has not been applied previously to the analysis of this amino acid, it is necessary to add an excess of the perchloric acid and titrate back with standard glycine solution. This procedure, described by Toennies and Callan (3), is necessary, since histidine forms a monoperchlorate and diperchlorate, the former being only slightly soluble in glacial acetic acid. It was found that histidine may be analyzed with fair precision and accuracy by the perchloric acid back titration technique. The purity of the purified *l*(-)-histidine was shown to be 100.13 per cent (average of six determinations with a probable error of the mean of 0.14) by the analysis of six samples ranging in weight from 0.0911 to 0.1418 gm.

The behavior of the basic amino acids in the formol titration has been investigated by Levy (4) who concluded that values about 7 per cent high are obtained with histidine under optimum conditions. The present authors' finding that the location and sharpness of the end-point vary with the concentration of formaldehyde is in agreement with the observation of

<sup>3</sup> Amino Acid Manufactures, Lot No. 8, A. P. grade.

<sup>4</sup> Amino Acid Manufactures, Lot No. 6, A. P. grade.

<sup>5</sup> Amino Acid Manufactures, Lot No. 12, A. P. grade.

<sup>6</sup> Amino Acid Manufactures, Lot No. 9, A. P. grade.

Levy. The maximum sensitivity (maximum value of  $\Delta E/\Delta V^7$  at the end-point of the titration) occurs at 0.3 M formaldehyde concentration. Under these conditions the apparent equivalent weight of histidine is  $100.9 \pm 0.1$  per cent of the theoretical value. Adjustment of the pH of the amino acid solution to any given value is unnecessary in the titration.

*Criteria of Optical Purity*—The solubility of *l*(-)-histidine in water was measured with large and small excesses of solute. Under these conditions, the presence of *d*(+)-histidine, *dl*-histidine, or other amino acids would be revealed by significant differences in the solubility values provided that mixed crystals were not formed.

Samples of the purified *l*(-)-histidine which were 7 per cent and 150 per cent in excess of the accepted solubility<sup>8</sup> (4.29 gm. per 100 gm. of water at 25° (5)) were placed in separate oil sample bottles. 40 ml. of distilled water were added to each bottle, the solutions were warmed to 50°, and the bottles were tightly stoppered and rotated in a thermostat at  $25.10^\circ \pm 0.05^\circ$ . At intervals, samples of each solution were drawn from the bottles by means of a 5 ml. pipette equipped with a cotton filter. Each solution was transferred to a weighed Petri dish and evaporated to constant weight at 95°. It was assumed that equilibrium had been attained when the weight of residual solid in successive 5 ml. volumes was constant. The solubility values found, 4.091 and 4.104 gm. per 100 gm. of solution, differ by only 0.34 per cent.

In a second series of determinations solubility was measured by formol titration with the glass electrode. In calculation of the solubility values from the experimental data account was taken of the observation, referred to previously in this report, that the apparent weight of histidine determined by formol titration is 100.9 per cent of the theoretical value. The corrected solubility values found with small and large excesses of solute, 4.115 and 4.127 gm. per 100 gm. of solution, differ by only 0.29 per cent. The average of the solubility values determined by the gravimetric and titrimetric methods, 4.098 and 4.121 gm. per 100 gm. of solution, differ by only 0.56 per cent.

In another method employed to establish the degree of purity of the purified *l*(-)-histidine, advantage was taken of the effect which any impurity would have on the specific rotation of a given sample of the histidine. The specific rotation of a sample (A) of the purified *l*(-)-histidine was determined at 25°. 3 gm. of this material were shaken at 35–40° with 25 ml. of distilled water and the suspension was rotated for 4 days in a thermostat at 25°. The suspension was filtered and the precipitate was washed

<sup>7</sup> The change in voltage per unit change in volume of base.

<sup>8</sup> The value, 4.19 gm. per 100 gm. of water at 25°, given by Dunn *et al.* (5), is in error.

data reported for this amino acid are accurate within a small percentage of probable error. The solubility of *l*(-)-histidine at  $25.10^\circ \pm 0.05^\circ$  was shown to be  $4.110 \pm 0.012$  gm per 100 gm. of aqueous solution and  $4.286 \pm 0.013$  gm. per 100 gm. of water. No solubility data with which to compare these figures could be found in the literature.

The average specific rotations of *l*(-)-histidine in water (Table I) were shown to be  $-38.95^\circ$  ( $0.06^\circ$ , probable error of the mean) at  $25.00^\circ \pm 0.02^\circ$  and  $-43.05^\circ$  ( $0.08^\circ$ , probable error of the mean) at  $0.46^\circ \pm 0.03^\circ$ . The

TABLE III  
*Specific Rotation Data for l(-)-Histidine in Water at 25°\**

Solute per 100 ml solution	Polarimetric tube length	Temperature	$\alpha$ (observed optical rotation)	$[\alpha]_D^t$	$[\alpha]_D^{25.0}$	Bibliographic reference No
gm	dm	°C	degrees	degrees	degrees	
2.08	0.5	20	-0.40	-38.46	-37.32	15
3.234	2.0	20	-2.57	-39.27	-38.12	16
0.775	2.0	20		-39.3	-38.16	17
3.55	1.0	20	-1.40	-39.44	-38.30	15
2.30	2.0	26	-1.75	-38.1	-38.33	18
2.22	0.5	20	-0.44	-39.65	-38.51	15
2.000	4.001	24.0	-3.121	-39.01	-38.81	†
0.752-3.770	4.000	25.0	-1.169 to -5.904	-38.95	-38.95	This paper

\* The specific rotation,  $[\alpha]_D^{25} = -40.70^\circ$  ( $c = 3.898$ ,  $l = 1$ ,  $\alpha = -1.45^\circ$ ), was reported by Bergmann and Zervas (23) in 1928. This paper was not found until after the present manuscript was in the proof form. It is apparent that the *l*(-)-histidine used by these investigators was of high purity and that the specific rotation,  $-40.34^\circ$  (corrected to  $25^\circ$ ), is higher than the value  $-38.95^\circ$  found by the present authors. It may be assumed, however, that the specific rotation of Bergmann and Zervas' histidine may have been as low as  $-38.97^\circ$  (at  $25^\circ$ ) if the maximum probable error in the observed rotation,  $-1.45^\circ$ , were  $0.05^\circ$ . It is of interest, also, that an  $[\alpha]_D = -39.74^\circ$  ( $l = 6$ ,  $c = 3.183$ ,  $\alpha = -7.59^\circ$ ) for *l*(-)-histidine was reported by Kossel and Kutsher (24) in 1899. The reliability of this value is uncertain, however, since neither the chemical purity of the *l*(-)-histidine sample nor the temperature at which the observed rotation was measured was stated.

† Dunn, M. S., and Stoddard, M. P., unpublished results.

most reliable data in the literature for the specific rotation of *l*(-)-histidine in water are given in Table III. The specific rotations reported by these investigators were corrected to  $25^\circ$  with the aid of the temperature coefficients, given in Table VI, which were derived from the data recorded in Table II.

It may be observed that, in every case, the values reported by the authors cited are smaller than that found in the present study. It is of further interest that (with the exception of the first value,  $-37.32^\circ$ )  $-38.45^\circ$  is the

average of all of the listed specific rotations. It is evident, therefore, that the accuracy of all of these values is relatively high and that *l*(-)-histidine of high purity is readily prepared by the fractional crystallization procedures commonly employed in the isolation of this amino acid from protein hydrolysates.

It may be shown that the observed optical rotations given in Tables I, IV, and V of *l*(-)-histidine in water, 42 weight per cent ethanol, and 6 *N* hydrochloric acid fall on, or very close to, straight lines relating  $\alpha$  and gm. of

TABLE IV

*Specific Rotations of l(-)-Histidine in Aqueous Solutions of Organic Solvents*

Solvent	$\rho$ (solute per 100 gm solution)	$c$ (solute per 100 ml solution)	Organic solvent	Density of solution	Temperature	$\alpha$ (observed optical rotation)	$[\alpha]_D^t$
	gm	gm	weight per cent		°C	degrees	degrees
Ethanol	1 004	0 920	42 3	0 916	25 00	-1 485	-40 35
	0 803	0 736	42 1	0 916	25 00	-1 200	-40 70
	0 610	0 551	42 1	0 904	25 00	-0 904	-41 00
	0 403	0 368	42 2	0 913	25 00	-0 601	-40 85
	0 313	0 250	42 1	0 923	0 80	-1 300	-43 40
	0 608	0 561	42 1	0 923	0 80	-0 980	-43 60
	0 403	0 374	42 2	0 928	0 80	-0 653	-43 65
	0 573	0 551	20 0	0 962	25 05	-0 860	-39 00
	0 578	0 561	20 0	0 971	1 00	-0 955	-42 50
	0 574	0 557	24 2	0 970	0 7	-1 011	-45 45
Methanol	0 572	0 553	24 2	0 967	17 8	-0 949	-42 45
	0 572	0 551	24 2	0 963	25 0	-0 923	-41 85
	0 572	0 551	24 2	0 963	25 3	-0 912	-41 40
	0 572	0 547	24 2	0 957	39 7	-0 861	-39 32
	0 572	0 545	24 2	0 953	57 1	-0 837	-38 35
	0 573	0 540	24 2	0 943	70 9	-0 824	-38 10
	0 594	0 551	41 9	0 928	25 0	-0 968	-43 92
Acetone	0 594	0 558	41 9	0 940	0 85	-1 093	-49 00
Dioxane	0 540	0 551	28 4	1 020	25 0	-1 155	-52 45
	0 540	0 555	28 4	1 028	0 75	-1 259	-56 60

solute per 100 ml. of solution in plots of these data. It may be concluded from this observation, as well as by an inspection of the data in these tables, that the specific rotation of *l*(-)-histidine does not vary materially with different concentrations of solute under the stipulated conditions.

It has been reported that the specific rotations of other amino acids are similarly unaffected by changes in concentration of solute. The amino acids investigated include *l*(-)-cystine in orthophosphoric acid, trichloroacetic acid, and 0.5 to 2.5 *M* hydrochloric acid (Andrews (8)), *l*(+)-arginine in water at the isoelectric pH, hydrochloric acid, and sodium hydroxide



TABLE V  
Specific Rotations of *l*(-)-Histidine in Acid Solutions

Solvent		<i>c</i> (solute per 100 ml. solution)	<i>d</i> (density of solution)	<i>p</i> (solute per 100 gm. solution)	Moles acid per mole histidine	<i>t</i> , temperature	$\alpha$ (observed optical rotation)	$[\alpha]_D^{25}$ (specific rotation)	Series
		gm.		gm.		°C.	degrees	degrees	
6.08	N HCl	1.0064	1.0986	0.9160	93.1	24.8	+0.537	+13.34	A ( <i>c</i> varied at constant temperature and acid concentration)
6.08	" "	1.525	1.1000	1.386	61.0	24.8	+0.815	+13.36	
6.08	" "	4.0533	1.1059	3.6653	22.6	24.8	+2.159	+13.32	
6.08	" "	1.540	1.1108	1.386	61.0	0.5	+0.547	+8.88	B ( <i>t</i> varied at constant <i>p</i> and acid concentration)
6.08	" "	1.531	1.1044	1.386	61.0	15.0	+0.709	+11.58	
6.08	" "	1.525	1.1000	1.386	61.0	24.8	+0.815	+13.36	
6.08	" "	1.519	1.0956	1.386	61.0	35.1	+0.923	+15.20	
6.08	" "	1.512	1.0912	1.386	61.0	45.1	+1.016	+16.80	
0.63	" "	9.726	1.0309	9.4345	1.000	24.8	+0.282	+0.73	C <sub>1</sub> ( <i>c</i> varied at constant temperature and molal ratio of acid to histidine)
0.239	" "	3.705	1.0105	3.6659	1.000	24.8	+0.287	+2.39	
0.095	" "	1.475	1.0021	1.4718	1.000	24.8	+0.150	+2.54	
0.0359	" "	0.5571	0.9992	0.5574	1.000	24.8	+0.065	+2.92	
0.0136	" "	0.2104	0.9981	0.2108	1.000	24.8	+0.021	+3	
1.026	" "	12.367	1.0589	11.678	1.29	24.8	+1.199	+2.42	C <sub>2</sub> ( <i>c</i> varied at constant temperature and molal ratio of acid to histidine)
0.592	" "	7.1271	1.0326	6.9018	1.29	24.8	+1.023	+3.59	
0.335	" "	4.032	1.0271	3.9257	1.29	24.8	+0.718	+4.45	
0.143	" "	1.7184	1.0060	1.7082	1.29	24.8	+0.369	+5.37	
0.0548	" "	0.6562	1.0007	0.6558	1.29	24.8	+0.145	+5.52	
1.026	" "	12.367	1.0589	11.678	1.29	24.8	+1.199	+2.42	D ( <i>c</i> and acid concentration varied at constant temperature)
0.532	" "	4.6913	1.0226	4.5878	1.76	24.8	+1.600	+8.53	
0.344	" "	1.7873	1.0096	1.7704	2.99	24.8	+0.852	+11.92	
0.272	" "	0.6658	1.0159	0.63836	6.34	24.8	+0.342	+12.83	
0.245	" "	0.2526	1.0027	0.25191	15.0	24.8	+0.126	+12.44	
0.097	" "	1.50			1.00	24.8	+0.163	+2.71	E* (acid concentration varied at constant <i>c</i> and temperature)
0.140	" "	1.50			1.29	24.8	+0.319	+5.32	
0.324	" "	1.50			3.13	24.8	+0.721	+12.02	
1.003	" "	1.6395	1.0203	1.6069	9.37	24.8	+0.834	+12.72	
4.51	" "	1.5033	1.0760	1.3971	46.0	24.8	+0.815	+13.55	
6.08	" "	1.525	1.1000	1.386	61.0	24.8	+0.815	+13.36	F (acid concentration varied at constant <i>c</i> and temperature)
8.0	" "	1.5003	1.1337	1.3234	80	24.8	+0.661	+11.01	
10.0	" "	1.5313	1.1719	1.3066	100	24.8	+0.432	+7.05	
0.594	M H <sub>2</sub> SO <sub>4</sub>	1.5126	1.0391	1.456	6.04	24.8	+0.838	+13.85	
2.14	" "	1.606	1.1324	1.418	20.4	24.8	+1.016	+15.82	
4.63	" "	1.492	1.2650	1.179	47.6	24.8	+1.063	+17.81	
9.04	" "	1.539	1.4885	1.034	90.1	24.8	+0.982	+15.95	
14.40	" "	1.496	1.7338	0.8628	148	24.8	+0.890	+14.87	

TABLE V—*Concluded*

\* The values +2.71, +5.32, and +12.02 and their dependent data were derived by interpolation from curves relating the figures given in Series C<sub>1</sub>, C<sub>2</sub>, and D.

(Miller and Andrews (9)), *l*(+)-glutamic acid in hydrochloric acid (Pertzoff (10)), and *l*(+)-lysine in hydrochloric acid (Lawrow (11)). On the other hand, Pertzoff (10) found that the specific rotation of *l*(+)-glutamic acid in an alkaline medium is linear in respect to the concentration of the amino acid, while that of *l*(+)-aspartic acid in both acid and alkaline media is linear with respect to the square root of the concentration of the amino acid. This dissimilar behavior of amino acids which resemble each other so closely in other respects is unexpected.

By comparable investigations with solutes other than amino acids, it has been shown that the specific rotations of certain optically active sugars, esters, and alcohols exhibit definite concentration effects, especially at high concentrations of solute. This phenomenon is illustrated by the observation of Clough (12) that there is a change of several hundred per cent in the specific rotation of *l*-methyl lactate over a concentration range of solute of 5 to 100 per cent.

The specific rotations at 25° of *l*(-)-histidine in 0.01 to 1 *N* hydrochloric acid solutions containing acid and histidine in a molal ratio of about 1:1 vary with respect to the concentration of the amino acid. It may be shown, however, by curves derived from plots of the data given in Table V, Series C<sub>1</sub> and C<sub>2</sub>, that this relationship is not linear. These concentration effects must necessarily be explained on some basis other than ionic dissociation, since there is negligible change in the proportion of the dicationic, monocationic, and zwitter ionic species. From a consideration of the acidic dissociation constants,  $pK_1 = 1.77$  and  $pK_2 = 6.10$ , of histidine at 25° and the curve (derived from the data in Table VII) relating the concentration of histidine monohydrochloride and pH of its aqueous solution, it may be calculated that the percentage distribution of total histidine among the three ionic species is 98.8, 0.6, and 0.6 in 0.626 *M* *l*(-)-histidine monohydrochloride and 98.6, 0.4, and 1.0 in 0.0645 *M* *l*(-)-histidine monohydrochloride for the monocation, the dication, and the zwitter ion, respectively.

It is noteworthy that the observed concentration effects of *l*(-)-histidine occur only in dilute hydrochloric acid solutions of widely varying ionic strength. In water, histidine exists primarily as the zwitter ion which contributes little to the ionic strength of the solution, while, in concentrated acid solutions, histidine is present almost entirely as the dication whose contribution to the ionic strength of the solution is masked by that of the acid. Further experiments on the influence of inorganic salts on the specific rotations of amino acids, suggested by these conclusions, are to be under-

TABLE VI  
Influence of Temperature on  $[\alpha]_D$  of *l*(-)-Histidine and Some Other Amino Acids\*

Amino acid	Solvent	$\rho$ (solute per 100 gm. solution) gm.	Temperature range ( $\lambda$ ) °C.	Temperature coefficient					Bibliographic reference No.
				(A)	15°	20°	25°	30°	
<i>l</i> (-)-Asparaginof	N HCl	8.042	14 -46	-0.125	-0.125	-0.125	-0.125	-0.125	19
"	Water	1.961	31 -60	-0.146				-0.180	19
"	"	7.407	38 -68	-0.137					19
<i>l</i> (+)-Aspartic acid	6.0 N HCl	1.820	0.7 -45.0	-0.077	-0.081	-0.078	-0.075	-0.073	†
"	1 N HCl	7.984	12 -41	-0.103	-0.085	-0.092	-0.099	-0.106	19
"	Water	0.990	14 -48	-0.112	-0.143	-0.133	-0.123	-0.113	19
"	"	1.872	32 -90	-0.097			-0.096	-0.092	20
"	"	1.961	48 -78	-0.127					19
"	0.2 N NaOH	2.584	18 -46	-0.182	-0.221	-0.215	-0.203	-0.188	19
"	1 N NaOH	6.00	17 -54	-0.049	-0.042	-0.043	-0.045	-0.047	19
"	1 " "	11.30	12 -40	-0.193	-0.202	-0.198	-0.194	-0.191	19
"	2 " "	19.76	12. -58	-0.180	-0.228	-0.214	-0.200	-0.187	19
<i>l</i> (-)-Cystine	1.02 N HCl	0.9797	20.54-30.25	-2.04	-2.04	-2.04	-2.04	-2.04	21
"	1.00 " "	1	20 -29	-1.7					8
<i>l</i> (+)-Glutamic acid	6.0 " "	1.815	0.7 -15.0	-0.061	-0.061	-0.061	-0.061	-0.061	†
"	1 N HCl	8.752	13 -43	-0.080	-0.078	-0.077	-0.077	-0.076	12
"	Water	0.99	15 -60	-0.076	-0.104	-0.099	-0.094	-0.086	12
"	"	2.91	44 -75	-0.081					12
<i>l</i> (-)-Histidine	6.08 N HCl	1.386	0.5 -45.1	+0.179	+0.184	+0.180	+0.176	+0.172	This paper
"	0.096 " " §	1.481	0 -45	+0.089	+0.091	+0.092	+0.088	+0.085	†
"	Water	0.551	18.4 -78.8	+0.089	+0.308	+0.257	+0.200	+0.158	This paper
"	Acetone (41.9 weight %)	0.591	0.89-25.0	+0.210				+0.102	"

<i>l</i> (-)-Histidine	Dioxane (28.4 weight %)	0.510	0.75-25.0	+0.171					This paper
"	Ethanol (20.0 weight %)	0.575	1.00-25.05	+0.146					"
"	Ethanol (42.1 weight %)	0.610	0.80-25.0	+0.099					"
"	Methanol (24.2 weight %)	0.572	0.7-70.9	+0.105	+0.166	+0.165	+0.162	+0.156	+0.116
<i>l</i> (-)-Leucine	12.0 N HCl	1.694	0-35	+0.045	+0.048	+0.047	+0.045	+0.042	+0.035
"	7.00 " "	1.80	0-45	+0.058	+0.060	+0.059	+0.057	+0.055	+0.049
"	6.00 " "	1.82	0-45	+0.062	+0.064	+0.063	+0.061	+0.059	+0.054
"	5.00 " "	1.84	0-45	+0.068	+0.068	+0.067	+0.065	+0.063	+0.058
"	4.00 " "	1.86	0-45	+0.075	+0.075	+0.073	+0.072	+0.069	+0.064
"	3.00 " "	1.89	0-45	+0.082	+0.085	+0.082	+0.080	+0.077	+0.072
"	2.00 " "	1.92	0-45	+0.091	+0.101	+0.095	+0.092	+0.087	+0.082
"	1.00 " "	1.94	0-45	+0.10	+0.13	+0.12	+0.11	+0.10	+0.10
"	Water	2.085	0-45	+0.064	+0.074	+0.066	+0.058	+0.050	+0.034
"	1.05 N NaOH	1.891	0-45	-0.087	-0.090	-0.087	-0.085	-0.084	-0.082
<i>l</i> (-)-Tyrosine	1.1 " HCl	5	16-26	-0.270	-0.386	-0.277	-0.241	-0.220	†
"	6 N HCl	4	16-26	-0.260	-0.306	-0.269	-0.233	-0.210	22

\* Numerous data on the influence of temperature on the specific rotations of amino acids at wave-lengths of light other than  $\lambda = 5893 \text{ \AA}$ . reported in the literature were not considered.

† The observed rotation of purified *l*(-)-asparagine dissolved in 6.08 N hydrochloric acid decreased from  $+2.383^\circ$  to  $+1.748^\circ$  in 34 hours at  $25^\circ$ . Since the specific rotation,  $+24.6^\circ$ , calculated (see below) for *l*(+)-aspartic acid is the accepted value (5), it seems certain that the asparagine was hydrolyzed to aspartic acid. It is not improbable, therefore, that asparagine may be hydrolyzed appreciably in 1 N hydrochloric acid at elevated temperatures.

$$[\alpha]_D^{25} = \frac{+1.748^\circ \times 100.0}{2.015 \times 132.1/150.1 \times 4.000} = +24.6^\circ \text{ in } 6.08 \text{ N HCl}$$

† Dunn, M. S., and Stoddard, M. P., unpublished data.

§ Normality of the acid in the amino acid solution.

|| Gm. of solute per 100 ml. of solution.

taken. An interpretation of the interesting data on the specific rotations of *l*(-)-histidine at varying concentrations of hydrochloric acid and sulfuric acid, given in Table V, Series E and F, is to be presented elsewhere.

The specific rotations of *l*(-)-histidine (Table IV) in 42.2 per cent ethanol, 24.2 per cent methanol, 41.9 per cent acetone, and 28.4 per cent dioxane are all different, even though these solvents have identical dielectric constants. It would appear, therefore, that the rotation of *l*(-)-histidine in solvents other than water is influenced inappreciably by the dielectric constant. On the other hand, the authors' experiments are consistent with the observation of Lowry (13) that the specific rotations of a number of optically active solutes in a series of solvents appear to be dependent upon the dipole moment of the pure solvent. The present authors have found that the specific rotations of *l*(-)-histidine increase linearly

TABLE VII  
*pH of Aqueous Solutions of l(-)-Histidine Monohydrochloride\**

Histidine monohydrochloride	pH
<i>moles per l.</i>	
0.600	3.95
0.191	4.00
0.153	4.01
0.122	4.01
0.061	4.01
0.0305	4.02
0.0158	4.09
0.0079	4.21
0.000	6.60

\* Amino Acid Manufactures, *l*(-)-histidine monohydrochloride monohydrate Lot No. 5, A. P. grade

with the decreasing average solvent dipole moments which were calculated from the dipole moments and the mole fractions of the components of the mixed solvents.

The rotation of *l*(-)-histidine in aqueous solutions of acetone is not in harmony with the foregoing observation. From a consideration of the polarity of acetone ( $\mu = 2.48$ ), it would be expected that the specific rotation of *l*(-)-histidine in 42 per cent acetone solution would be considerably less than that in water. It was assumed that this apparent abnormality might be explained by a reaction of histidine with acetone analogous to that of this and other amino acids with formaldehyde. In order to test this hypothesis, the rotation of *l*(-)-histidine in water as a function of acetone concentration was measured by the apparatus and technique described in Paper VII of this series (14). It was found, however, that the rotation of

histidine decreases regularly as acetone is added to its aqueous solution. Although there is no evidence from this experiment of complex formation, it cannot be regarded as settled that optical activity of amino acids is not a function of solvent polarity. An effect similar to that observed in the present experiments was reported by Clough (12) who found that the rotation of *l*-methyl aspartate in benzene, chloroform, methanol, and water decreased regularly, while the rotation in acetone was similar to that in chloroform.

According to Lowry (13), concentration effects are explained by the orienting influence of the optically active molecules of the solute upon one another. Since maximum orientation of molecules occurs in the solid (crystalline) state, it may be inferred that the rotations exhibited by increasing concentrations of an optically active solute in different solvents should approach the same value. The observation that there is no appreciable change in the specific rotation of certain amino acids with varying, but relatively low, solute concentration may be explained if there is negligible interaction between solute molecules.

Pertzoff (10) advanced the view that concentration effects are due to variations in the electrical field of the solvent, resulting from changes in the effective molar volume of the solute. That the latter is a function of glutamic acid and aspartic acid concentrations was demonstrated by Pertzoff who observed that the maximum change in the molar volume and in the optical rotation with concentration occurs with the ion,  $^+\text{NH}_3\text{R}(\text{COO}^-)_2$ .

The effect of temperature upon the specific rotation of amino acids has been studied by a number of workers. The results of these investigations are summarized in Table VI. The temperature coefficients at 15°, 20°, 25°, 30°, and 40° were determined by interpolation of curves derived from plots of the data given by the authors cited. It is of interest that temperature coefficients of the amino acids are both positive and negative, increase and decrease in magnitude with increasing temperature, and (with the exception of the large value, -2.04, for *l*(-)-cystine) range from -0.042 to +0.308 at 15° and +0.034 to -0.187 at 40°.

#### SUMMARY

1. It has been shown that *l*(-)-histidine of 99.5 per cent or higher purity may be prepared by the fractional crystallization from water and alcohol of material isolated by standard methods from protein hydrolysates.

2. The solubility of *l*(-)-histidine at  $25.10^\circ \pm 0.05^\circ$  is  $4.286 \pm 0.013$  gm. per 100 gm. of water. The specific rotation (*l* 4 dm. and  $\lambda$  5893 Å.) of *l*(-)-histidine<sup>9</sup> in water is  $-38.95^\circ \pm 0.06^\circ$  at  $25.00^\circ \pm 0.02^\circ$  (*c* 0.752 to

<sup>9</sup> The value  $+39.01^\circ$ , given by Dunn *et al.* (5) as the specific rotation of *l*(-)-histidine in water at 25°, is in error.

3.770) and  $-43.05^\circ \pm 0.08^\circ$  at  $0.46^\circ \pm 0.03^\circ$  ( $c$  0.765 to 3.826) and in 6.08  $N$  hydrochloric acid is  $+13.34^\circ \pm 0.02^\circ$  at  $24.80^\circ \pm 0.05^\circ$  ( $c$  1.0064 to 4.053).

3. The specific rotation of  $l(-)$ -histidine in water, aqueous ethanol, and 6  $N$  hydrochloric acid does not vary materially with different concentrations of solute. In 0.01 to 1  $N$  hydrochloric acid solutions at constant molal ratio (1:1) of acid to solute, the specific rotation of  $l(-)$ -histidine is dependent upon the concentration of the solute, although the function is not linear.

4. The specific rotations of  $l(-)$ -histidine in solvents other than water have been found to be dependent upon the dipole moment, but not the dielectric constant, of the solvent.

5. Temperature coefficients of  $l(-)$ -histidine and certain other amino acids have been compared.

6. Hypotheses advanced to explain the described physicochemical behavior of  $l(-)$ -histidine have been discussed.

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# THE FATE OF DIETARY SERINE IN THE BODY OF THE RAT\*

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The discovery that phosphatidylserine is a normal component of certain cephalins (1, 2) suggested the present investigation of the relationship of dietary serine to the various nitrogenous bases that occur in phosphatides. In a previous report (3), the metabolic interrelations of ethanolamine, choline, glycine, and betaine were studied by feeding each of these substances, labeled with isotopic nitrogen, to rats under standard conditions, and determining the isotopic composition of the ethanolamine and choline which were obtained from the phosphatides of their bodies. It was shown that ethanolamine and choline were rapidly incorporated into the body phosphatides and that ethanolamine was continuously and rapidly methylated to choline, under the conditions of the experiment. This latter reaction has since been shown to proceed unimpaired even under conditions of diet which might, *a priori*, have been supposed to be unfavorable (4).

The present feeding of isotopic serine was conducted under conditions in all respects similar to those of the earlier report (3), and the analytical figures obtained are therefore comparable with those previously published. In the feeding of an isotopic metabolite, it is always desirable to isolate the substance fed from the tissues of the animal. The difficulties of isolating serine from the small amounts of material available were circumvented by the collection of the ammonia liberated from suitably purified materials by the action of periodate, which reaction appears to be characteristic of vicinal hydroxyamino compounds (5). Thus, in working up the nitrogenous compounds obtained on hydrolysis of the mixed body phosphatides of the rats that had been fed isotopic serine, after extraction of ethanolamine and choline from CaO (6), 21 mg. of N remained unextracted, and this material was fairly rich in isotope. As acids in general should resist extraction from CaO by organic solvents, any of the unextracted N that might be liberated by periodate may be assumed to arise from serine. About 4 mg. of N were so obtained and proved to be much richer in isotope than any other fraction of the phosphatides, which was taken to support the assumption that it was indeed serine nitrogen.

The isotope content of this fraction indicates that at least 6 per cent of

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all the serine present in the phosphatides was derived from the serine of the diet. The figure actually must have been somewhat higher, as the isotopic serine fed was diluted by serine present in the casein of the diet.

That serine may be decarboxylated biologically has been previously suggested (1, 7). This idea finds support in the present observation of an appreciable concentration of isotope in the ethanolamine of the phosphatides. The analytical figure indicates that at least 2.6 per cent of all the ethanolamine in the phosphatides was formed by the decarboxylation of dietary serine. If the serine decarboxylated was of the same isotopic composition as that isolated from the phosphatides, 100(2.6/6.0) or 43 per cent of all the ethanolamine present in the phosphatides was prepared in this fashion in 3 days. These figures are the same order of magnitude as those previously reported for the conversion of glycine to ethanolamine under similar conditions (3).

The fact that the serine fraction, in the present experiment, had a higher concentration of  $N^{15}$  than the ethanolamine is taken to disprove the possibility suggested by Folch and Schneider (1) that ethanolamine might be nothing but a postmortem artifact, resulting from the decarboxylation of phosphatidylserine after death. Such a hypothesis would require that the serine and ethanolamine have identical isotopic composition.

The finding of isotope in the choline from the phosphatides is not surprising in view of the previously established methylation of ethanolamine. The choline isolated in the present experiment was somewhat less than half as rich in  $N^{15}$  as the ethanolamine, a finding in close agreement with that previously reported when ethanolamine labeled with heavy nitrogen was fed (3).

In comparing the metabolic pathways of *d*- and *l*-leucine, Ratner, Schoenheimer, and Rittenberg (8) observed that the biologically abnormal isomer contributed more of its nitrogen to the urine and less to the body proteins than did the normal *l*-leucine. Further, they showed that, when *d*-leucine labeled with  $N^{15}$  was fed, the urinary ammonia was exceptionally rich in isotope. In the present feeding of *dl*-serine, the relatively large amounts of isotope excreted in the urine and the small amount retained in the organ proteins may similarly be due to the body's inability to operate economically upon the *d* isomer. The high isotope concentration in the urinary ammonia may also be ascribed to *d*-serine (9), but no particular significance can be attached to the urinary "serine" fraction, as there was perceptible spillage of food into the urine bottles.

In order to determine whether there was incorporation of dietary serine into the organ proteins, the ammonia liberated by periodate was analyzed. It was of course recognized that this nitrogen was not derived exclusively from serine. If milk proteins be taken as characteristic of mammalian

proteins in general, fully half of this material may have been derived from threonine (10); so that, had pure serine been isolated, it would probably have been found to contain twice as high an isotope concentration as the mixed "hydroxyamino acids" that were actually analyzed.

The possibility that the 3-carbon chain of cystine might be derived from serine has been clearly stated by Toennies (11). This idea is also implicit in the recent results of du Vigneaud and collaborators who showed that the unsymmetrical thio ether S-( $\beta$ -amino- $\beta$ -carboxyethyl)homocysteine served as a source of cystine in the intact animal (12) and as a precursor of cystine in the presence of liver slices (13). Cystine isolated in the present experiment from the organ proteins of rats fed serine had an isotope content 5 per cent of that in the serine fed and 5 times as high as that of the glutamic acid isolated from the same source. This concentration of  $N^{15}$  is too high to be accounted for by transamination and indicates that the 3-carbon chain of serine is more or less directly converted to cystine in the body of the rat. It is particularly gratifying to us to learn that Binkley and du Vigneaud (14) have detected essentially the same conversion in the presence of liver slices.

#### EXPERIMENTAL

*Preparation of Isotopic dl-Serine*—Ethyl phthalimido acetate was prepared from isotopic potassium phthalimide according to the method of Schoenheimer and Ratner (15) and the product hydrolyzed under the conditions suggested by these authors. Glycine was not isolated as such but was directly esterified with ethanol in the presence of dry HCl (16) after removal of water. Glycine ethyl ester hydrochloride was benzoylated by refluxing with benzoyl chloride in dry benzene (17) and the ethyl hippurate recrystallized from ether-petroleum ether. M.p. 59–61°.

Ethyl hippurate was condensed with formic ester and the product reduced with aluminum amalgam in wet ether, without intermediate isolation, according to the procedure of Erlenmeyer and Stoop (18). N-Benzoylserine ethyl ester was crystallized from benzene by addition of petroleum ether. The product melted sharply at 80°. Serine was liberated by hydrolysis with 7 per cent HCl and, after removal of benzoic and hydrochloric acid, was precipitated from water by addition of ethanol. The glistening white platelets were filtered off and washed with ethanol.

$C_3H_7NO_3$ . Calculated, N 13.3; found, N (Kjeldahl) 13.3;  $N^{15}$  = 1.985 atom % excess

*Feeding Experiment*—The same basal diet was used in this experiment as in those previously reported (3). Each of three male adult rats, average weight 252 gm., was fed 15 gm. of basal diet + 1.5 mm of isotopic dl-serine per day for 3 days. In this period there was an average weight gain of

5 gm. The rats were killed and their bodies worked up essentially as in the previous study. Only innovations in the procedure will be given here.

The urines from each day were pooled and aliquots taken for total N, ammonia, and urea determination (19). Recrystallized dioxanthidyl urea was prepared for isotope analysis. "Serine" N was determined by titration

TABLE I  
*Isotopic Composition of Substances Isolated*

Three adult male rats were fed 1.5 mm of isotopic *dl*-serine per rat per day for 3 days, equivalent to 189 mg. of N in all. The isotope content of the fractions analyzed was computed on the basis of 100 atom per cent in the serine fed, and on this basis the analytical error of the N<sup>15</sup> determinations is about  $\pm 0.15$  per cent.\* The N<sup>15</sup> content of the serine fed was 1.985 atom per cent excess.

Source of compound	Compound isolated	Total nitrogen	N <sup>15</sup> content	Isotope recovered
		mg.	atom per cent <sup>a</sup>	mg.
Urine, 1st day	Total N	679.0	3.0	20.4
" 2nd "	" "	752.0	3.5	26.3
" 3rd "	" "	669.0	4.7	31.5
" 3rd "	Urea	567.0	3.0	17.0
" 3rd "	Ammonia	31.9	29.2	9.3
" 3rd "	"Serine"†	7.2	67.9	4.9
Total phosphatides	Total N	41.8	2.5	1.0
	Ethanolamine		2.6	
	Choline		1.2	
	Unextracted N‡	21.3	2.5	0.5
	"Serine"†	3.9	6.0	0.2
Organ proteins	Total N	1995.0	1.2	23.9
	Glutamic acid		1.0	
	Cystine		5.1	
	Amide N		1.9	
	"Hydroxyamino acids"†		2.4	

\* The values tabulated =  $\frac{\text{atom } \% \text{ N}^{15} \text{ in compound isolated}}{\text{atom } \% \text{ N}^{15} \text{ in serine fed}} \times 100$ .

† The material analyzed was the NH<sub>3</sub> liberated by the action of periodate after removal of preformed NH<sub>3</sub>.

‡ The material analyzed was the water-soluble residue that remained on CaO after ethanolamine and choline had been extracted with ether and ethanol respectively.

of the ammonia liberated by the action of periodate (20), after removal of preformed ammonia. This same ammonia evolved on periodate oxidation was employed for isotope analysis.

The mixed body phosphatides of the rats were isolated as before (3) and further purified by an additional acetone precipitation from petroleum ether solution. After acid hydrolysis the water-soluble fraction was de-

posited on CaO, from which ethanolamine and choline were successively extracted as before with ether and ethanol (6). The nitrogenous material that resisted extraction from CaO was shaken out with water, calcium ion being removed by treatment with oxalic acid. That fraction of the nitrogen that is designated "serine" in Table I is the portion of the unextracted N that was liberated as ammonia after oxidation by periodate.

The cuprous mercaptide of cysteine was isolated from the proteins of the internal organs according to Graff, Maculla, and Graff (21). Copper was removed as the sulfide and the cysteine oxidized by aeration in slightly alkaline solution. On concentration and acidification with acetic acid, cystine precipitated out and was once recrystallized. Although it was still not ash-free, analysis of a trial run indicates that it was not contaminated with extraneous nitrogen.

$C_3H_{12}N_2O_4S_2$ . Calculated, S 26.7, N 11.7; found, S 25.3, N 11.0; S:N = 1.004

A neutralized aliquot of the protein hydrolysate was treated with potassium carbonate and the amide ammonia was aerated into acid. The residue was then treated with periodate and the liberated  $NH_3$  was designated "hydroxyamino acid" N in Table I.

Other isolations were carried out as in the previous report (3).

#### SUMMARY

*dl*-Serine, containing  $N^{15}$ , has been prepared and fed to rats in small amounts for 3 days under standard conditions of diet. Dietary serine has been shown to be incorporated as such into the body phosphatides and the proteins of the organs.

Evidence is presented for the decarboxylation of serine to give ethanolamine in the animal body.

From the finding of a very high isotope content in the cystine isolated from the proteins of the organs, it is concluded that the carbon chain of serine is converted into cystine in the body of the rat.

The author wishes to thank Mr. I. Sucher for the isotope analyses included in the present report.

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# THE FORMATION OF CYSTEINE FROM HOMOCYSTEINE AND SERINE BY LIVER TISSUE OF RATS\*

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Previous communications from this laboratory have presented evidence that the unsymmetrical thio ether, *ll*-S-( $\beta$ -amino- $\beta$ -carboxyethyl)homocysteine (cystathionine),<sup>1</sup> may serve in lieu of cystine for the growth of rats (2) and that an enzyme is present in the liver capable of cleaving the thio ether to yield cysteine (3). Brand, in his original suggestion (4) regarding this thio ether as an intermediate in the formation of cystine from methionine, visualized the addition of homocysteine to aminoacrylic acid or its peptides with subsequent cleavage of the thio ether so formed. The hypothesis of an intermediate thio ether formation was in contrast with that of Nicolet (5) who suggested that H<sub>2</sub>S was split from homocysteine and was added to aminoacrylic acid to form cysteine. Toennies (6), on the other hand, suggested that methionine, through addition to serine, formed the methyl sulfonium derivative of the S-( $\beta$ -amino- $\beta$ -carboxyethyl)-homocysteine, which, by removal of methyl alcohol followed by cleavage, would yield cysteine.

If the thio ether were the true intermediate between methionine and cystine, it seemed to us that it might be possible to bring about the conversion of homocysteine to the thio ether by allowing liver tissue of rats to act on a mixture of homocysteine plus a compound which might reasonably be expected to yield aminoacrylic acid. Since rat liver contains a system capable of cleaving the thio ether to cysteine, as we have already shown, formation of the thio ether would thus lead to the production of cysteine from homocysteine. Consequently, *dl*-homocysteine and *dl*-serine were shaken anaerobically for 2½ hours with slices of rat liver. Considerable extra cystine was found to be present in the neutralized,

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Through a private communication from Dr. Stetten, it was learned that he had obtained results which afford complementary and independent proof of this finding that serine is involved in cysteine synthesis. His results are presented in the accompanying paper (1).

<sup>1</sup> This thio ether will undoubtedly be the subject of various investigations and because of the cumbersomeness of the chemical name we suggest *cystathionine* as its common name.

aerated trichloroacetic acid filtrate of the digest. In later experiments as much as 3.6 mg. of cysteine were found to be produced from 10 mg. of *dl*-homocysteine. When homocysteine without added serine was treated under these same conditions, only a very small production of cysteine was observed to have taken place. Apparently a precursor for the carbon chain of the cysteine to be formed must be made available.

When pyruvic acid and ammonia, which may be considered in equilibrium with aminoacrylic acid, were tried with homocysteine under the same conditions as with the serine, no cysteine production beyond the control levels could be detected. The possibility therefore arises that serine may react directly with homocysteine to form the thio ether instead of first being converted to aminoacrylic acid. This question should be readily answerable by proper labeling of the serine with deuterium, a type of approach we now have under way.

A saline extract prepared from rat livers had almost the same ability to produce cysteine from homocysteine and serine as the liver slices. When the digestions with either saline extract or liver slices were carried out in an atmosphere of oxygen, the amount of cysteine produced was greatly diminished.

In the present experiments with homocysteine as well as in the earlier experiments with the thio ether (3), strongly positive tests for the presence of hydrogen sulfide were obtained at the end of the digestion periods. Cyanide in a concentration of 0.01 *M* has been shown to inhibit the production of hydrogen sulfide from cysteine (7). Cyanide has also been utilized in the studies of the enzymatic cleavage of the thio ether (3) and it was found that hydrogen sulfide production was inhibited with little if any decrease in cysteine production. In the present series of experiments cyanide was found to inhibit hydrogen sulfide production but did not appreciably decrease the amount of cysteine produced from the homocysteine.

*d*-Homocysteine was found to be ineffective in leading to the formation of cysteine by liver tissue. *d*-Serine likewise failed to yield appreciable amounts of extra cysteine when used in place of the *dl*-serine. It may be pointed out that when *dl*-homocysteine and *dl*-serine were used the cystine isolated from the digest was found to have the *l* configuration. Apparently the isomers of the *d* configuration cannot be used to any great degree by liver tissue for the production of cysteine. On the other hand, Dyer and du Vigneaud (8) have shown that the *d* and *l* isomers of homocysteine are of equal efficacy in the replacement of cystine on a cystine-deficient diet.

When methionine was treated in the same manner as the homocysteine, little or no cysteine was produced. It would therefore appear that homo-

cysteine under these conditions was considerably more effective than methionine. Obviously, homocysteine, to go to cysteine, does not have to go by way of methionine as would be the case if the Toennies theory were correct. Thus the results are consistent with the view that the demethylation of methionine to homocysteine is an intermediary step in the conversion of methionine to cysteine. This result favors the theory of Brand, or a slight modification thereof, rather than that of Toennies for the mechanism of cysteine formation from methionine.

#### EXPERIMENTAL

The experiments with homocysteine were conducted in much the same manner as those reported with the mixed thio ether (3). The homocysteine and serine were dissolved in 25 cc. of Krebs-Ringer solution (9). In the early experiments 40 mg. of *dl*-homocysteine were used with 50 mg. of *dl*-serine and 300 to 400 mg. of liver tissue (dry weight). In later experiments the amount of *dl*-homocysteine was reduced to 20 mg. and finally to 10 mg. The yield of cysteine from the smaller amounts of homocysteine was much higher.

Liver slices were used in most of the experiments presented here. A saline extract prepared from fresh rat liver had almost the same activity as the liver slices in cysteine production but no further attempt has been made to purify the enzyme or enzymes involved. Anaerobic conditions (an atmosphere of 95 per cent nitrogen and 5 per cent carbon dioxide) were used in these experiments. 50 mg. of *dl*-serine were added to each digest. Cystine was determined in the neutralized, aerated, trichloroacetic acid filtrates by the method of Sullivan and Hess (10). The results of a series of experiments are given in Table I.

Samples digested for  $2\frac{1}{2}$  hours under an atmosphere of 95 per cent oxygen and 5 per cent carbon dioxide contained only 0.9 mg. of cysteine when 20 mg. of *dl*-homocysteine were used or 0.6 mg. of cysteine when 10 mg. of *dl*-homocysteine were used with added serine. Samples digested anaerobically for  $2\frac{1}{2}$  hours with no added serine contained an average of 0.3 mg. of cysteine. The amounts of liver used in these control experiments were of the same order of magnitude as with the examples recorded above.

The digestion of 20 mg. of *dl*-homocysteine with 50 mg. of *dl*-serine and 370 mg. of liver tissue in the presence of 0.01 M NaCN led to the production of 2.6 mg. of cysteine. This value is only slightly lower than with those digests containing no cyanide and, therefore, little or no inhibition of the enzyme or enzymes must have occurred. Hydrogen sulfide production was completely inhibited. Pyruvic acid and ammonia were ineffective in promoting cysteine production from homocysteine.

Since the homocysteine and the serine used in these experiments were



both of the *dl* modification, it became important to determine whether the *d* isomers are capable of entering into the reactions. In a representative experiment 50 mg. of *d*-serine and 10 mg. of *dl*-homocysteine were digested with 290 mg. of liver slices for 2½ hours. Only 0.7 mg. of cystine was found to be present in the filtrate. In another experiment the filtrate from the digestion of 10 mg. of *d*-homocysteine with 50 mg. of *dl*-serine and 340 mg. of liver tissue for 2½ hours contained a total of only 0.4 mg. of cystine.

TABLE I

*Cysteine Produced from Homocysteine and Serine by Liver Tissue*

50 mg. of *dl*-serine were present in each digest. The temperature of incubation was 38°.

Experiment No.	Liver dry weight	Amount of <i>dl</i> -homocysteine	Time	Cysteine produced	H <sub>2</sub> S production
	mg.	mg.	hrs.	mg.	
I4	370	40	2½	2.3	+++
I5	340	40	2½	2.0	+++
Y2	320	40	2½	2.4	+++
Y3	300	40	2½	2.3	+++
Z2	420	40	2½	2.4	+++
Z3	340	40	2½	2.4	+++
AB2	370	20	1	1.7	++
AB3	360	20	1	1.6	++
Z1	340	20	2½	3.1	+++
Z5	350	20	2½	2.9	+++
AB4	310	20	5	4.1	+++
AB5	370	20	5	4.3	+++
AC1	340	20	10	5.0	+++
AC2	320	20	10	5.1	+++
AC3	420	10	1	1.4	++
AC4	370	10	1	1.7	++
AC5	360	10	2½	3.0	+++
AF1	320	10	2½	3.1	+++
AF2	320	10	2½	3.1	+++
AF3	330	10	5	3.4	+++
AF4	320	10	5	3.6	+++

In order to determine the optical properties of the cysteine formed from the homocysteine, the specific rotation of cystine isolated from a digest was measured. 250 mg. of *dl*-homocysteine and 1.5 gm. of *dl*-serine dissolved in 250 cc. of Krebs-Ringer solution were digested 12 hours with 30 cc. of a saline liver extract in the presence of 0.01 M NaCN and under an atmosphere of nitrogen and toluene. About 90 mg. of cystine were indicated in the filtrate by the Sullivan method. The cuprous mercaptide of cysteine was precipitated by the addition of cuprous chloride. The cuprous mercap-

tide was decomposed with hydrogen sulfide. The hydrogen sulfide was removed from the filtrate by aeration and the cysteine was oxidized with 0.01 N iodine solution. 49 mg. of material were recovered from the solution by isoelectric precipitation. The Sullivan method indicated a 90 per cent content of cystine. The Folin-Marcenzi method as modified by Kassell and Brand (11) indicated a 99 per cent content of disulfide calculated as cystine. The presence of a small amount of some disulfide compound other than cystine, probably homocystine, was thus indicated. 32 mg. of the material dissolved in 5 cc. of 1 N HCl gave a specific rotation value of  $[\alpha]_D^{22} = -195^\circ$ .

The experiments with methionine were performed in a manner exactly analogous to those with homocystine. The filtrates from the digestion of 10 mg. of *l*-methionine and 50 mg. of *dl*-serine for  $2\frac{1}{2}$  hours with  $300 \pm 20$  mg. of liver tissue contained an average of 0.4 mg. of cystine. Samples to which no methionine was added contained 0.3 mg. of cystine. These results on methionine under these conditions in which homocystine was effective were repeatedly confirmed. Further experiments are under way to see whether conditions can be altered in liver slice experimentation to bring about higher yields of cystine when methionine is employed.

#### SUMMARY

The conversion of a mixture of homocystine and serine to cystine by liver tissue of rats has been demonstrated. In the absence of serine only slight cystine formation was observed to have taken place. Homocystine was found to be far more effective than methionine. The possible significance of these findings in conjunction with the biological behavior of the unsymmetrical thio ether, *ll*-S-( $\beta$ -amino- $\beta$ -carboxyethyl)homocystine (cystathionine), has been discussed.

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# ADIPIIC ACID AS AN OXIDATION PRODUCT OF THE DIAMINO-CARBOXYLIC ACID DERIVED FROM BIOTIN\*

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In recent communications we have established the molecular formula as well as the nature of the functional groups of biotin (3-5). We now wish to report experiments which give information regarding the carbon skeleton of this compound. The diaminocarboxylic acid,  $C_9H_{18}O_2N_2S$ , which we had obtained by the treatment of biotin with  $Ba(OH)_2$  at  $140^\circ$  served as the starting material. This compound upon oxidation with alkaline potassium permanganate at room temperature was found to yield an ether-soluble acidic oxidation product which crystallized from ether in small prisms and melted at  $152-153^\circ$ . This product was identified as adipic acid by the neutral equivalent, by a mixed melting point, and by the preparation of the diamide and the di- $\beta$ -naphthylamide. The quantity of adipic acid that we were able to isolate in pure form from the oxidation products amounted to about 20 per cent of that theoretically possible. Adipic acid was also obtained in a somewhat higher yield by oxidation of the diaminocarboxylic acid with nitric acid. The isolation of the same compound under both these oxidizing conditions minimizes to a great extent the possibility of a rearrangement to an intermediate which could have yielded the adipic acid. Thus the consistent formation of adipic acid as an oxidation product of biotin may be interpreted in one of two possible ways. Either biotin contains an aliphatic side chain which is capable of yielding adipic acid or the latter has its origin in a cyclic structure which is cleaved by the oxidation. In the first case one of the carboxyl groups of the adipic acid must be the carboxyl group originally present in biotin, and it should therefore be possible, by the oxidation of a derivative of the diaminocarboxylic acid in which the carboxyl group has been eliminated, to decide between the two alternatives. After several attempts by other methods the objective was achieved by a Curtius degradation. In this way the carboxyl group was replaced by an amino group. Biotin methyl ester was

\* Preliminary reports of this work have been published (1, 2). The authors wish to express their appreciation to the S. M. A. Corporation for a research grant which has aided greatly in this work. They also wish to thank Mr. W. O. Frohring and the Research Staff of the S. M. A. Corporation and Dr. R. Major and the Research Staff of Merck and Company, Inc., for supplies of biotin.

converted into the hydrazide,  $C_{10}H_{18}O_2N_4S$ . When the calculated amount of nitrous acid was added to this in dilute HCl, a rather stable azide was formed which, when dissolved in absolute ethanol and boiled for 2 hours, was transformed into the corresponding ethyl urethane,  $C_{12}H_{21}O_3N_3S$ . The further breakdown of the urethane was performed in two ways.

The first route was the complete hydrolysis with strong  $Ba(OH)_2$  which led to the triamine,  $C_8H_{19}N_3S$ , characterized as the sulfate, the tripicolonate, and the tribenzoyl derivative.

The second route was a stepwise degradation first with strong HCl to the monoamine,  $C_9H_{17}ON_3S$ , followed by the drastic  $Ba(OH)_2$  treatment to the triamine,  $C_8H_{19}N_3S$ , which was identical with the triamine above. Evidently the alkaline hydrolysis opens the urea ring and cleaves the urethane group to yield the triamine; the acid treatment, however, splits the urethane selectively, without affecting the urea ring, to give the monoamine. The latter corresponds to biotin with the carboxyl group replaced by an amino group. Subjection of this compound to alkaline treatment opens the urea ring to yield the triamine.

The triamine was subjected to the same oxidation procedures which we employed for the oxidation of the diaminocarboxylic acid. In the first experiment 10 mg. of the triamine were oxidized with nitric acid. No indication of the presence of adipic acid among the ether-soluble oxidation products was observed. A repetition of the experiment on the same amount of material led to a similar result. Under the same conditions adipic acid had been readily isolated after the oxidation of similar amounts of the diaminocarboxylic acid.

Finally when more material became available, 50 mg. of the triamine sulfate were oxidized with potassium permanganate under the same conditions employed in the oxidation of the diaminocarboxylic acid. Here again no trace of adipic acid could be detected in the ether-soluble oxidation products, although the amount of adipic acid which might have been formed from the relatively large amount of triamine used would have made its isolation and identification comparatively easy. The absence of adipic acid in isolable amounts among the oxidation products of the triamine therefore affords substantial evidence that one of the carboxyl groups of the adipic acid formed by oxidation of the diaminocarboxylic acid is identical with the original carboxyl group of biotin. This means in effect that the 6-carbon moiety giving rise to adipic acid upon oxidation is not present in biotin as a cyclic structure, but indicates the presence of an aliphatic acid side chain in biotin which is capable of yielding adipic acid on oxidation. The possible interpretation of these findings and other data previously published (1, 3-7) with respect to the structure of biotin has been discussed in the preliminary note (2) and will not be repeated here.

## EXPERIMENTAL

*Oxidation of Diaminocarboxylic Acid with Nitric Acid*—10 mg. of the diaminocarboxylic acid sulfate were dissolved in 1 cc. of nitric acid (sp. gr. 1.42). After the first vigorous reaction the solution was heated on the steam bath for 1 hour and was then evaporated to dryness *in vacuo*. The residue was taken up in 1 cc. of water and was evaporated to dryness again, this process being repeated twice to free the products from excess nitric acid. The residue was then continuously extracted with ether for several hours. The ether extracts were washed with a small amount of water, were dried over sodium sulfate, and were evaporated. The resulting 3 mg. of acids were washed with a few drops of ether and were purified by sublimation *in vacuo* (100°, 0.01 mm.). The sublimate was recrystallized from ether and yielded 1 mg. of short prisms melting at 152–153°. No depression of the melting point was observed when this material was mixed with a sample of pure adipic acid.

*Oxidation of Diaminocarboxylic Acid with Barium Permanganate*—20 mg. of the diaminocarboxylic acid sulfate were dissolved in 2 cc. of water and 1.2 cc. of 0.1 N Ba(OH)<sub>2</sub> and were oxidized at room temperature by adding dropwise 1.8 cc. of 0.1 M barium permanganate. The solution was kept at room temperature overnight and the MnO<sub>2</sub> was removed by filtration and was washed repeatedly with hot water. The clear filtrate was concentrated to a small volume *in vacuo*, was acidified to Congo red with N H<sub>2</sub>SO<sub>4</sub>, and was extracted with ether for several hours. 3 mg. of crude acids, melting at 120–130°, were obtained from the ether extract. The acids were purified by sublimation *in vacuo* and the fraction subliming at 80–100° (0.01 mm.) was further purified by crystallization from ether. 1 mg. of short prisms melting at 152–153° was obtained. The substance gave no depression of the melting point when mixed with a sample of pure adipic acid.

*Diamide of Adipic Acid from Biotin*—1 mg. of adipic acid from biotin was transformed into its acid chloride by treatment with 5 drops of thionyl chloride. The acid chloride was distilled *in vacuo* and the distillate was mixed with a solution of ammonia in water. After standing for 10 minutes the solution was evaporated to dryness and the residue was washed with ice water. The latter was then sublimed at 150–160° (0.01 mm.). The purified material melted at 224–226°, with a change at 210° from short prisms to long needles. A sample of adipic acid amide prepared in the same manner from authentic adipic acid behaved exactly as described for the product from biotin and a mixture of the two compounds showed no depression of the melting point.

*Di- $\beta$ -naphthylamide of Adipic Acid from Biotin*—1 mg. of the acid was transformed into the acid chloride as described and this was coupled with

purified  $\beta$ -naphthylamine in ether. The ether was evaporated and the crystalline residue was transferred to a filter with  $N$  HCl and was washed with  $N$  HCl and water. The material was further purified by crystallization from glacial acetic acid, followed by sublimation *in vacuo* at  $255^{\circ}$  (0.01 mm.). The pure material melted at  $267$ – $268^{\circ}$  and likewise showed a transformation from prisms to needles at  $240$ – $250^{\circ}$ . No depression of the melting point was observed when this compound was mixed with a sample of authentic adipic acid di- $\beta$ -naphthylamide, which melted at  $267$ – $268^{\circ}$  and showed the change from prisms to needles at  $250^{\circ}$ .

*Oxidation of Diaminocarboxylic Acid with Alkaline Potassium Permanganate*—50 mg. of the diaminocarboxylic acid sulfate were dissolved in 2 cc. of  $N$  NaOH and to this solution was added dropwise with stirring a 5 per cent solution of potassium permanganate until the violet color remained for 10 minutes. The reaction mixture was then heated on the steam bath for 10 minutes to destroy the excess permanganate, the manganese dioxide was removed by filtration, was washed with hot water, and the filtrate was acidified to Congo red with concentrated HCl. The acidic aqueous solution was then extracted for 48 hours with ether and from the ether extract 17 mg. of crude acids were obtained. From the above acids it was possible to isolate 4 mg. of adipic acid, m.p.  $147$ – $150^{\circ}$ , by crystallization from ethyl alcohol. From the mother liquors 1 mg. more of adipic acid was obtained. The adipic acid was converted into its di- $\beta$ -naphthylamide as described and 7 mg. of pure di- $\beta$ -naphthylamide melting at  $266$ – $267^{\circ}$  were obtained.

$C_{26}H_{24}O_2N_2$ .	Calculated.	C 78.76, H 6.10
(396.45)	Found.	" 78.88, " 6.03

*Biotin Hydrazide*—20 mg. of biotin methyl ester and 0.5 cc. of hydrazine hydrate were heated in a sealed tube at  $130^{\circ}$  for 3 hours. The clear solution was then evaporated to dryness *in vacuo* and the crystalline residue was washed with methanol and ether. 19 mg. of material melting at  $237$ – $239^{\circ}$  were obtained which on recrystallization from water gave clusters of prisms which melted at  $238$ – $240^{\circ}$ .

$C_{10}H_{10}O_2N_4S$ .	Calculated.	C 46.50, H 7.02, N 21.68
(258.3)	Found.	" 46.82, " 7.09, " 21.88

*Ethyl Urethane,  $C_{12}H_{21}O_3N_3S$* —52 mg. of biotin hydrazide were dissolved in 0.5 cc. of  $N$  HCl and the solution was cooled with ice. To this was added 0.4 cc. of a solution containing 200 mg. of  $NaNO_2$  in 5 cc. of water and the solid precipitate of the azide was collected, was washed with ice water, and was dried over  $P_2O_5$  at room temperature. The dried azide (39 mg.) was boiled for 2 hours with 5 cc. of absolute ethanol and the solution was then evaporated to dryness *in vacuo*. 40 mg. of crude urethane were obtained which sintered at  $165^{\circ}$  and melted at  $171$ – $176^{\circ}$ . 10 mg. of the compound

were purified by sublimation at 165–170° (0.01 mm.). The sublimed material melted at 188–190°.

$C_{17}H_{21}O_3N_3S$ .	Calculated.	C 50.16, H 7.37, N 14.62
(287.4)	Found.	" 50.53, " 7.64, " 14.19

*Preparation of Amine Hydrochloride,  $C_9H_{17}ON_3S \cdot HCl$* —66 mg. of crude urethane were dissolved in 3 cc. of concentrated  $HCl$  and the solution was heated on the steam bath for 2 hours. The dark brown solution was then evaporated to dryness *in vacuo* and the residue was taken up in water and evaporated again to free it from excess  $HCl$ . The material was then taken up in 2 cc. of water, was decolorized with a small amount of charcoal, and the clear solution was evaporated to dryness *in vacuo*. The crystalline residue was purified by crystallization from dilute ethanol. 32 mg. of needles melting at 265–270° with decomposition were obtained. From the mother liquors another 10 mg. of material were obtained which melted with decomposition at 260–270°.

$C_9H_{17}ON_3S \cdot HCl$ .	Calculated.	C 42.95, H 7.20, N 16.68, Cl 14.03
(251.74)	Found.	" 43.15, " 7.17, " 16.80, " 14.20

*Triamine,  $C_8H_{19}N_3S$* —The sulfate of the triamine was prepared from the amine hydrochloride. 12 mg. of the latter were hydrolyzed in the usual manner with  $Ba(OH)_2$ . 11 mg. of the triamine sulfate were obtained which melted at 249–252° with decomposition.

$(C_8H_{19}N_3S)_2 \cdot 3H_2SO_4 \cdot 2H_2O$ .	Calculated.	N 11.85, S 22.62
(708.81)	Found.	" 11.57, " 22.74

The sulfate of the triamine was also obtained from the urethane. 39 mg. of the urethane were hydrolyzed with  $Ba(OH)_2$  and the reaction product isolated as described for the preparation of the diaminocarboxylic acid sulfate. The 35 mg. of sulfate obtained could be crystallized from a mixture of water and methanol. The pure material melted at 249–252° with decomposition.

The picrolonate of the triamine,  $C_8H_{19}N_3S$ , was prepared by mixing an aqueous solution of the sulfate with a saturated solution of picrolonic acid. The yellow picrolonate, which decomposed at 250°, was purified by crystallization from aqueous ethanol.

$C_8H_{19}N_3S \cdot 3C_{10}H_9O_2N_4$ .	Calculated.	C 46.49, H 4.41, S 3.27
(981.8)	Found.	" 46.19, " 4.50, " 3.14

The tribenzoyl derivative of the triamine was prepared as follows: 10 mg. of the triamine sulfate were dissolved in 1 cc. of water and were benzoylated by shaking with an excess of benzoyl chloride and  $N KOH$ . The oily tribenzoyl derivative that separated was extracted with chloroform. The chloroform solution was washed with  $N KOH$ , 2  $N HCl$ , and water, was dried over sodium sulfate, and was evaporated to dryness. The tribenzoyl



derivative was obtained as clusters of prisms which when crystallized from methanol and ether melted at 194–195°.

$C_{25}H_{41}O_2N_3S$ .	Calculated.	C 69.44, H 6.23, N 8.37
(501.6)	Found.	" 68.99, " 6.41, " 8.01

The tribenzoyl derivative was also prepared from the pierolonate. 22 mg. of the pierolonate were dissolved in 15 cc. of hot water, the solution was acidified to Congo red with dilute HCl, and the pierolonate was decomposed with wool. The wool was separated and was washed repeatedly with hot water and the filtrates and washings were concentrated to a volume of 1 cc. *in vacuo*. The solution was then benzoylated as described above and the benzoyl derivative isolated and crystallized. 5 mg. of benzoyl derivative were obtained, which melted at 194–195°.

*Oxidation of Triamine*—50 mg. of the triamine sulfate were oxidized with potassium permanganate as described for the oxidation of the diamino-carboxylic acid. 10 mg. of crude ether-soluble oxidation product were obtained. Fractionation of this material by the procedures used for the purification of the crude adipic acid fractions yielded no adipic acid. The same negative results were obtained in our earlier experiments on smaller amounts of material with nitric acid as the oxidizing agent, as stated in the preliminary note.

The authors wish to express their appreciation to Dr. Julian R. Rachele of this laboratory for carrying out the microanalyses.

#### SUMMARY

Oxidation of the diaminocarboxylic acid derived from biotin, either with nitric acid or with permanganate, yields adipic acid as one of the oxidation products.

That one of the carboxyl groups of the adipic acid is the original carboxyl group in the biotin molecule is indicated by the non-formation of adipic acid on oxidation of the amine formed by a Curtius rearrangement of biotin hydrazide.

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# SIMPLIFIED BROMIDE DETERMINATION IN BLOOD AND URINE\*

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In 1938 Brodie and Friedman (1) published a method for the determination of bromide in tissues and biological fluids. Tissues, serum, urine, or spinal fluid was dried and then fused with sodium hydroxide on a sand bath and the melt containing the bromide was dissolved in water.

The use of bromide as a measure of the extracellular fluid was demonstrated by Brodie, Brand, and Leshin (2, 3). In the present paper, a method for determining bromide in the serum and the urine is reported. The fusion, with subsequent solution of the melt and transferring, is the most complicated and time-consuming step of the original method. A study was made of different protein precipitants to substitute for the fusion, and the use of trichloroacetic acid was found to give quantitative recoveries of added bromide to serum.

The filtrate from trichloroacetic acid is treated essentially as in the original method. The bromide is oxidized to bromate by sodium hypochlorite buffered with acid phosphate. The excess hypochlorite is reduced by sodium formate and the addition of iodide to the bromate in acid solution results in the liberation of 6 equivalents of iodine. The iodine is then titrated with standard thiosulfate solution.

A simplified and accurate method for the determination of bromide in urine is also included in this communication. The bromide and chloride are precipitated from urine as the silver salts in the presence of nitric acid (4). The supernatant fluid is removed, the halides are suspended in acid phosphate solution, and the bromides oxidized as for serum.

## Reagents—

Trichloroacetic acid, 10 per cent.

Sodium dihydrogen phosphate ( $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ ), 40 per cent.

Sodium hypochlorite, 1.0 N in about 0.1 N NaOH. Pass chlorine gas with constant stirring into a solution containing 44.8 gm. of NaOH in 1000 ml. of solution. The alkalinity is tested at intervals by destroying the hypochlorite in 1 ml. of solution with 2 ml. of 3 per cent hydrogen peroxide, diluted to 10 ml. and titrated with 0.1 N HCl. The titer should be between 0.8 and 1.2 ml. The reagent is stable for several weeks in the refrigerator.

\* A preliminary report of this paper was presented at the meeting of the American Society of Biological Chemists at Boston, April, 1942.

Sodium formate, 50 per cent.

Potassium iodide, 20 per cent.

Ammonium molybdate, 10 per cent.

Silver nitrate, 7.5 per cent.

Nitric acid, 1 per cent.

Sodium thiosulfate, 0.005 N. This is made from a stock 0.1 N solution and standardized before use.

Starch, 1 per cent.

### *Procedure*

*Serum*—Into a 125 ml. Erlenmeyer flask put 5 ml. of serum and to it add with constant shaking 25 ml. of 10 per cent trichloroacetic acid. Shake thoroughly with a clean rubber stopper, let stand 15 minutes, and filter through a dry 9 cm. Whatman No. 1 filter paper, refiltering the first portion. In this manner about 25 ml. of filtrate are obtained. 10 ml. of filtrate, equivalent to 1.67 ml. of serum, are transferred to a 125 ml. Erlenmeyer flask and the trichloroacetic acid is neutralized by the addition of 0.5 gm. of sodium bicarbonate. Now add 5 ml. of 40 per cent sodium dihydrogen phosphate and 8 ml. of 1.0 N sodium hypochlorite. The resulting solution is immersed in a boiling water bath for 10 minutes, at the end of which time the excess hypochlorite is reduced by the addition of 5 ml. of 50 per cent sodium formate. The sides of the flask are washed with water, and the flask shaken and replaced in the bath for 5 minutes. The solution is cooled to room temperature, 10 ml. of 6 N sulfuric acid added, and the flask further cooled in an ice bath to about 10°. Add 0.3 ml. of ammonium molybdate and 1 ml. of 20 per cent potassium iodide. After standing for 1 minute the liberated iodine is titrated with 0.005 N sodium thiosulfate. The starch-iodine titration end-point is difficult to recognize in natural light but results can be duplicated to 0.01 ml. of 0.005 N thiosulfate by placing the burette in a box with the inside coated with white paint. The box is illuminated from an upper back corner with a 60 watt lamp and the end-point is observed by holding the flask against the side of the box opposite the lamp. A blank determination is run under identical conditions, with 8 ml. of 10 per cent trichloroacetic acid and 2 ml. of water for the 10 ml. of filtrate. The titration of this blank is subtracted from the volume of thiosulfate in the determination. 1 ml. of 0.005 N sodium thiosulfate is equivalent to 0.0667 mg. of Br. The thiosulfate is standardized by measuring accurately a 0.01 N bromate solution into a 125 ml. Erlenmeyer flask, made to 20 ml. with water, and 5 ml. of sodium dihydrogen phosphate, 5 ml. of 50 per cent sodium formate, and 10 ml. of 6 N sulfuric acid are added in the order named. Cool to 10°, add 1 ml. of 20 per cent KI, 0.3 ml. of 10 per cent ammonium molybdate, and titrate the liberated iodine with sodium thiosulfate.

*Calculations*—Since 1 ml. of 0.005 N thiosulfate is equivalent to 0.0667 mg. of Br, and an aliquot of 1.67 ml. of serum is used, the calculation may be made as follows:

$$(\text{Titration} - \text{blank}) \text{ ml.} \times \frac{\text{thiosulfate N}}{0.005} \times 0.0667 \times 60 = \text{mg. \% Br}$$

The thiosulfate normality is known and the last three factors of the equation may be combined into one, giving the calculation directly from the titration. Thus, when the normality of the thiosulfate is exactly 0.005 N, 1 ml. is equivalent to 4.0 mg. per cent of Br.

*Urine*—Add 5 ml. of urine to a 50 ml. graduated conical centrifuge tube and bring the volume to 25 ml. with distilled water. Add 3 ml. of concentrated nitric acid and then with constant stirring add dropwise 7.5 per cent silver nitrate until no further precipitate forms. Overlay the solution with 95 per cent ethyl alcohol to lower the surface tension and prevent the formation of a silver halide film which forms during centrifugation. Let stand 20 minutes and then centrifuge at a moderate speed for 10 minutes. Siphon or decant the supernatant fluid and wash the precipitate with 20 ml. of 1 per cent nitric acid, and centrifuge again. The precipitate is next suspended with the aid of a stirring rod in 5 ml. of 40 per cent acid phosphate to which 10 ml. of sodium hypochlorite are added. The centrifuge tube is placed in a boiling water bath for 10 minutes and the suspension stirred at frequent intervals by vertical motion, after which time 5 ml. of sodium formate are added and the tube kept in the water bath 5 minutes longer. The solution is then filtered through a moistened 7 cm. Whatman No. 1 filter paper and the precipitate washed several times with water. To the filtrate are added 10 ml. of 6 N sulfuric acid, cooled in an ice bath, and after the addition of 1 ml. of 20 per cent KI and 0.3 ml. of 10 per cent ammonium molybdate the solution is titrated as in the serum determination. A blank is run with each series of determinations. The blank consists of 5 ml. of acid phosphate and 10 ml. of hypochlorite, and the solution is treated under identical conditions as for the urine determination. It is not necessary to filter the blank.

It is not possible to oxidize the bromide in urine directly, owing to the presence of interfering substances that react with hypochlorite. Isolating the silver halides makes the method applicable for urines of any composition. The normal excretion of bromide can be determined with a fair degree of accuracy by analyzing 10 ml. of urine. This amount of urine normally contains about 0.025 mg. of Br. When the chloride concentration in the urine is low, the addition of 2 ml. of 1 per cent NaCl should be made to 5 ml. of urine. The same amount of salt is also added to the blank.

Proteins in urine are removed by the addition of 15 ml. of 10 per cent trichloroacetic acid with constant stirring to 15 ml. of urine. The solution

is placed in a boiling water bath for 1 minute, cooled for 30 minutes, and filtered until clear. 10 ml. of the filtrate, equivalent to 5 ml. of urine, are determined as described.

*Calculations*—The blank is subtracted from the determination and the difference in titration is due to the amount of bromide in 5 ml. of urine.

$$(\text{Titration} - \text{blank}) \text{ ml.} \times \frac{\text{thiosulfate N}}{0.005} \times 0.0667 \times 20 = \text{mg. \% Br}$$

TABLE I  
*Recoveries of Bromide Added to Serum*

Bromide added	No. of analyses	Average Br recovered	Extreme range recovered	Average recovery
mg.		mg.	mg.	per cent
0.049	6	0.0490	0.0480-0.0500	100.0
0.079	8	0.0782	0.0075-0.0790	99.0
0.197	11	0.1961	0.1950-0.1990	99.5
0.493	10	0.4904	0.4870-0.4940	99.5

TABLE II  
*Comparison of Recoveries by Fusion Method (I) and Trichloroacetic Acid Precipitation*

Determination No.	Fusion method	Trichloroacetic acid method	Determination No.	Fusion method	Trichloroacetic acid method
	mg. per cent	mg. per cent		mg. per cent	mg. per cent
1	13.1	13.3	5	15.6	15.4
	13.1	13.2		15.5	15.5
2	11.6	11.9	6	13.6	13.7
	11.7	11.9		13.6	13.8
3	18.6	18.8	7	19.5	19.6
	18.3	18.7		19.4	19.4
4	16.5	16.5	8	20.4	20.6
	16.6	16.5		20.3	20.4

### Results

*Serum*—Amounts of bromide from 0.150 to 1.480 mg. were added to 5 ml. of normal serum, equivalent in amounts to 3 to 30 mg. per cent. The blank consisted of the same serum without added bromide. In Table I are shown the amounts added to the equivalent of 1.67 ml. of serum and the recoveries.

A series of determinations was made to find whether bromide in the circulating serum could be determined in the same manner as the bromide added to serum *in vitro*, as shown in Table I. A number of human sub-

jects were given from 3 to 5 gm. of sodium bromide and several hours later blood was withdrawn and aliquot determinations were made in duplicate by the trichloroacetic acid filtration and the original fusion method. The results of some of these are shown in Table II. Amounts of bromide added to serum *in vitro* were always recovered quantitatively by the trichloroacetic acid method, and the results checked favorably with those obtained by the fusion method in a large series of patients given bromide. However, in a few instances the fusion method yielded somewhat higher results. This discrepancy (less than 15 per cent) occurred in serum from diabetics taken when the patient was not in a fasting state. No explanation can be given at the present time as to why the bromide could not be filtered quantitatively in these exceptional cases. This problem is being studied further.

TABLE III  
*Recoveries of Bromide Added to Urine*

Bromide added	No. of analyses	Average Br recovered	Extreme range recovered	Average recovery
mg.		mg.	mg.	per cent
0.148	5	0.141	0.139-0.144	95.3
0.237	8	0.223	0.220-0.224	94.1
0.592	8	0.568	0.562-0.571	96.0
0.829	9	0.787	0.774-0.801	95.0
1.480	8	1.392	1.376-1.407	94.0
2.960	4	2.771	2.750-2.787	93.6

*Urine*—The recovery of bromide from urine is somewhat less than quantitative, though constant, as is seen from Table III. No further bromide could be recovered from the residue.

#### SUMMARY

A technique for the determination of bromide in serum is described. The proteins are precipitated with trichloroacetic acid, the bromide oxidized to bromate, and the latter determined iodometrically.

The bromide in urine is determined by precipitating the silver halides and oxidizing the bromide in the suspended salts.

I wish to express my appreciation to Dr. Louis R. Ferraro, Pathologist at Fordham Hospital, for his encouragement in making this work possible, and to Professor George B. Wallace and Dr. Bernard B. Brodie of New York University for their valuable suggestions.

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# AZOLESTERASE\* ACTIVITIES OF ELECTROPHORETICALLY SEPARATED PROTEINS OF SERUM

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It has been shown previously that differentiation of members of the group of enzymes that hydrolyze esters of nitrogen alcohols is possible to some degree by differences in their occurrence in the blood of various species (2). Thus, horse and human sera contain cholinesterase and tropacocainesterase, but no demonstrable atropinesterase or cocainesterase. Differentiation between the former two may be based on the fact that cholinesterase occurs in erythrocytes but tropacocainesterase does not, and between the latter two on the finding that atropinesterase occurs in the sera of only some rabbits, while we have found cocainesterase in the sera of both those with and without atropinesterase. In order to obtain data that might serve more adequately to characterize these enzymes, and that might possibly be useful for their separation, determinations of the activities of the azolesterases in question were carried out on electrophoretically separated proteins of human, horse, and rabbit sera.

## EXPERIMENTAL

Two samples of fresh rabbit serum were used: Serum A was the pooled sera of four animals having an average atropinesterase activity of 132 units per 0.1 ml., and Serum B was the serum of a single rabbit having 336 units per 0.1 ml. The human serum was collected from six normal individuals, and the pooled material was used within a few days. The horse serum had been stored at 2–4° for 18 months, but had been sterilized by filtration and sealed while it was fresh.<sup>1</sup>

The electrophoretic method and apparatus have been described elsewhere (3, 4). Before being subjected to electrophoresis, the samples of rabbit and

\* The term azolesterase has been recently suggested by Glick (1) to designate the class of esterases that act on nitrogen alcohol esters in order to differentiate this group from other types of esterase. The azolesterases act not only on esters of the amino alcohol variety, but also on the nitrogen heterocyclic alcohol type; the demarcation between these two types remains to be clarified.

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<sup>1</sup> We are indebted to Dr. W. G. Maleolm of Lederle Laboratories, Inc., for the sample of normal horse serum.



human sera were dialyzed in viscose sausage casings against 3 liters of buffer consisting of the bicarbonate-Ringer solution used in the enzyme measurement without the  $\text{CaCl}_2$  (0.025 M  $\text{NaHCO}_3$ , 0.10 M  $\text{NaCl}$ , 0.025 M  $\text{KCl}$ , pH 8.2). The calcium salt was omitted in order to prevent the precipitation of carbonate that occurs when the solution is not in equilibrium with at least 5 per cent  $\text{CO}_2$ . The same buffer was used to fill the electrode vessels of the apparatus. To obtain a higher yield of  $\alpha$ - and  $\beta$ -globulins, the sample of horse serum was subjected to the following procedure: A 300 ml. sample was brought to one-third saturation with  $(\text{NH}_4)_2\text{SO}_4$ . The precipitate, principally  $\gamma$ -globulin, was discarded (5). The supernatant was then brought up to one-half saturation with  $(\text{NH}_4)_2\text{SO}_4$ . The precipitate, consisting mainly of  $\alpha$ - and  $\beta$ -globulins (5), was separated from the supernatant, washed, centrifuged, dialyzed against running water, and redissolved in a phosphate buffer (0.02 M  $\text{Na}_2\text{HPO}_4$  + 0.15 M  $\text{NaCl}$ , pH 7.5). It was then dialyzed against three changes of the same buffer for 3 days, the last being used to fill the electrode vessels of the electrophoresis apparatus. This sample contained all the electrophoretic components of normal horse serum but the relative amount of  $\alpha$ - and  $\beta$ -globulins had been considerably enhanced.

Since albumin has the greatest mobility and  $\gamma$ -globulin the lowest at these pH values, pure samples of albumin and  $\gamma$ -globulin could be obtained at each end of the protein column. Mixtures of albumin and  $\alpha$ -globulin free of  $\beta$ - and  $\gamma$ -globulins, and of  $\beta$ - and  $\gamma$ -globulins free of albumin and  $\alpha$ -globulin could also be obtained. This first separation was made in a large cell of 100 ml. capacity. The sample of albumin and  $\alpha$ -globulin was again dialyzed against the buffer to be used in the electrode vessels and the proteins were separated in a cell of 10 ml. capacity. In like manner  $\beta$ - and  $\gamma$ -globulins were separated.

The protein concentration of each separated component was determined by means of a Zeiss interferometer which had previously been calibrated by micro-Kjeldahl nitrogen determinations. This method provided a means of determining concentrations to an accuracy of about 3 per cent and did not consume any of the material.

The azolesterase measurements were carried out by the manometric method previously described, and the unit of activity employed was the same as that previously defined (2); *i.e.*, the quantity of enzyme required to liberate 1 c.mm. of  $\text{CO}_2$  in 300 minutes at  $30^\circ$  in a total volume of 4 ml. containing 0.25 per cent substrate (in the case of acetylcholine chloride 0.375 per cent was used). Actually the reactions were followed for only 120 minutes and extrapolations to 300 minutes were made. 1 ml. of undiluted protein solution was used in the side arm of a Warburg vessel with 3 ml. of substrate in the main chamber. Enzyme values, employed to express concentration, were defined as the number of units per 100 mg. of protein.

## DISCUSSION

From Table I it is apparent that the azolesterases studied cannot be separated from one another by electrophoretic means, since all of these enzymes appear predominantly in the  $\alpha$ - and  $\beta$ -globulin fractions. It would also appear from these data that there is no parallel between the electrophoretic homogeneity of the proteins and their enzyme activities. Thus, even though the  $\alpha$ - and  $\beta$ -globulins have been distinctly separated according to electrophoretic criteria, each enzyme appeared in both fractions in every instance with the exception of tropacocainesterase in rabbit Serum A. However, this work shows that the enzymes are most con-

TABLE I  
*Azolesterase Actions of Serum Protein Fractions*

Enzyme material		Protein concentration	Acetylcholinesterase	Atropinesterase	Tropacocainesterase	Cocainesterase
		mg. per ml	units per 100 mg.	units per 100 mg.	units per 100 mg.	units per 100 mg.
Rabbit Serum A	Dialyzed serum	53.2	2,500	1,200	280	690
	$\alpha$ -Globulin	0.61	23,000	21,000	3,300	10,800
	$\beta$ -Globulin	2.10	15,000	1,400	0	1,400
	$\gamma$ -Globulin	3.20	2,800	0	0	0
	Albumin	20.0	550	300	130	0
Rabbit Serum B	$\alpha$ -Globulin	1.50	10,700	24,700	2,600	3,700
	$\beta$ -Globulin	1.20	16,700	5,400	2,500	2,900
	$\gamma$ -Globulin	1.30	3,100	0	2,300	1,900
	Albumin	2.90	510	1,900	0	0
Horse serum	$\alpha$ -Globulin	15.3	5,600		470	
	$\beta$ -Globulin	8.5	4,900		410	
	$\gamma$ -Globulin	12.0	690		0	
	Albumin	5.2	0		0	
Human serum	$\alpha$ -Globulin	0.08	100,000		44,000	
	$\beta$ -Globulin	0.10	85,000		25,000	
	$\gamma$ -Globulin	0.23	0		0	
	Albumin	1.06	1,400		0	

centrated in their respective  $\alpha$ -globulin fractions, with the exception of cholinesterase in rabbit Serum B.

The purification of cholinesterase in horse serum had been undertaken previously with salting-out and adsorption techniques by Stedman and Stedman (6). They found that the enzyme was chiefly associated with the protein fraction soluble in 35 per cent  $(\text{NH}_4)_2\text{SO}_4$  and precipitated by 40 per cent salt concentration in a slightly acid medium. This pseudoglobulin fraction of serum corresponds to the  $\alpha$ -globulin separated by electrophoresis. McMeekin (7) obtained effective purification of the enzyme in horse serum by precipitation with lead acetate at pH 7.4. He reported that

very little enzyme was obtained with the protein preprecipitated by half saturation with  $(\text{NH}_4)_2\text{SO}_4$ . It has been the experience of the authors that the most highly purified enzyme preparation is obtained from the protein preprecipitated between one-third and one-half saturation with  $(\text{NH}_4)_2\text{SO}_4$ . The present experiments demonstrate that while most of the activity may be found in the  $\alpha$ -globulin of horse serum, the  $\beta$  fraction also is rich in cholinesterase, the  $\gamma$ -globulin contains very little, and the albumin none.

Since the chief purpose of this work was the separation of the enzymes, the degree of purification effected by electrophoresis was given secondary consideration. However, the data for rabbit Serum A demonstrate the relative concentrations of the enzymes in the different fractions. In the  $\alpha$ -globulin a purification was obtained of 9 to 10 times for acetylcholinesterase, 17 to 18 times for atropinesterase, 11 to 12 times for tropacocainesterase, and 15 to 16 times for cocainesterase.

#### SUMMARY

Acetylcholinesterase, atropinesterase, tropacocainesterase, and cocainesterase in serum could not be separated from one another by electrophoresis, although some purification of the enzymes could be produced by this means. The azolesterase concentrations were greatest in the  $\alpha$ - and  $\beta$ -globulin fractions, with the former generally exceeding the latter.

The authors wish to express their appreciation to Miss Helen Sikorski and Mrs. Vernon Strub for their technical assistance in the electrophoretic separations.

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# ACID-BASE EQUILIBRIUM IN THE NORMAL\*

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The pH<sub>s</sub> was determined on the arterial blood of normal young men by the gasometric method with a degree of sensitivity indicated for the glass electrode by Haugaard and Lundsteen (1). The data indicate a narrower range of pH for the arterial blood and for the tissues than that which one is led to expect from work reported heretofore.

## Methods

The work was done under basal conditions and novocaine anesthesia. The appearance and overt activity of the subject, the pulse, blood pressure, and depth, rate, and regularity of respiration were observed throughout the test and showed little or no change.

Arterial blood was collected without contact with air (or oil in the majority of instances), chilled immediately, and kept at 0-5° except when being equilibrated at the subject's temperature (rectal).

Carbon dioxide dissociation curves (two, three, four, or five points) of oxygenated whole blood and true plasma were determined, in general, as outlined by Dill in the appendix to Henderson's book (2). The  $p\text{CO}_2$  of content blood, and the plasma  $\text{CO}_2$  content were gotten by plotting (respectively) the blood  $\text{CO}_2$  content on the curve of whole blood and the content  $p\text{CO}_2$  on the curve of true plasma (on large scale log-log paper). The content  $p\text{CO}_2$  was also calculated (by use of the equation for a straight line). In the later experiments, in place of using true plasma curves, the plasma  $\text{CO}_2$  content was determined directly on the plasma of content blood which had been centrifuged under oil. The findings agreed well with values obtained with true plasma curves as above.

To calculate the pH<sub>s</sub>, constants appropriate to the temperature of equilibration (36.6-37.4°) were used. It was assumed that the change in solubility of  $\text{CO}_2$  in serum with change in temperature was comparable to that in water. Van Slyke's factor of 0.0671 for 38.0° (3) was used as a basis for calculating the factors for other temperatures. Values for  $pK'$  were derived from Van Slyke's value of 6.105 at 38.0° (confirmed by Dill's

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work at 37°) on the basis of a decrease of 0.005 per degree increase in temperature (4-6); *i.e.*, a decrease of 0.0005 per tenth of degree.

Work was done to determine the effect of the glycolytic formation of lactic acid during equilibration. It was concluded that, while by not correcting one permits this effect to introduce a slight artificial variability among the several cases, one does not necessarily, by correcting, decrease this variability, and in fact, because of unavoidable technical error in correcting, may accentuate it. Because of this and because we were interested not in absolute values for the normal, but in comparative values, it was decided not to correct our data.

For the same reasons no correction for the effect of the slight degree of oxygen unsaturation of the arterial blood as determined was made.

To approximate more closely the absolute value for  $pH_i$ , one must add 0.02 to the values given in this paper to correct for these combined effects.

It was demonstrated that the *in vitro* formation of  $CO_2$  in blood took place at such a slow rate as not to be measurable within the period of time taken to complete the work on a given blood.

To test the sensitivity of the method three separate specimens of blood were simultaneously collected on each of four subjects, the  $pH_i$ , etc., being determined in triplicate on each subject. The points used to get the three separate dissociation curves for each subject were used in combination to describe a six point dissociation curve. This was considered the standard curve for this subject. The standard blood  $CO_2$  content for each subject was obtained by averaging all the separate Van Slyke determinations of the blood  $CO_2$  content of each of the three specimens of blood (nine determinations). The standard plasma  $CO_2$  content for the subject was obtained in the same manner. The standard curve and blood and serum  $CO_2$  contents were used to derive the standard  $pH_i$ . The deviation of the values obtained on each of the three specimens of blood from the standard values for the subject was considered the error. From the average of the errors on all twelve experiments, the range of error, indicating the degree of sensitivity of the method, was obtained.

The pertinent data of the experiments on the three specimens of blood of one of the four subjects are given in Table I and in Fig. 1 as a representative sample. A digest of the findings will be given in the text.

It was found that the values for  $pH_i$  obtained with heparin from the Connaught Laboratories were higher than those obtained with heparin from Hynson, Wescott and Dunning, Inc. To be certain that this difference was attributable to the difference in heparin, two separate specimens of blood, one in which Connaught heparin and another in which Hynson heparin was used, were simultaneously collected on each of five subjects. In each instance the  $pH_i$  was at least 0.013 higher with the Connaught heparin. This was attributable practically entirely to the fact that the

blood dissociation curve was higher (and hence, since the blood  $\text{CO}_2$  content was the same, the  $p\text{CO}_2$  lower) with Connaught heparin and but slightly to the fact that the plasma  $\text{CO}_2$  content was higher.

The  $\text{pH}_s$  was determined in triplicate on one subject with Connaught heparin in concentrations 1, 2, and 3 times that ordinarily used. The

TABLE I  
*Representative Data to Indicate Sensitivity of Method*

The  $\text{pH}_s$ , etc., were obtained on three specimens of blood taken simultaneously from the same artery.

Specimen	Blood $\text{CO}_2$ content, vol. per cent			Plasma $\text{CO}_2$ content, vol. per cent			$p\text{CO}_2$ , mm.		$\text{pH}_s$	
	Analyses	Average	Deviation from standard	Analyses	Average	Deviation from standard		Deviation from standard		Deviation from standard
A	50.48	50.59	0.06	59.47	59.52	0.18	43.56	0.16	7.387	0.000
	50.63			59.56						
	50.67			60.08						
B	50.48	50.54	0.01	59.92	59.86	0.16	43.75	0.03	7.388	0.001
	50.32			59.89						
	50.83			59.78						
C	50.58	50.45	0.08	59.25	59.54	0.16	43.87	0.15	7.384	0.003
	50.45			59.54						
	50.33			59.82						
Standard...		50.53			59.70		43.72		7.387	

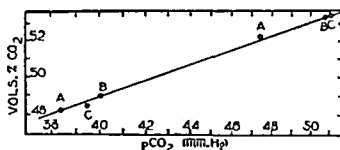


FIG. 1. Representative data to indicate the sensitivity of the method. Pairs of points are used to establish the  $\text{CO}_2$  dissociation curve for each of three specimens of blood, A, B, and C, taken simultaneously from the same artery. The curve shown is the "standard" curve plotted on a log-log scale. See the text.

variations in the values obtained were within our range of error and not in any given direction.

#### *Statement of Findings*

The range of error demonstrated was  $\pm 0.06$  volume per cent for blood  $\text{CO}_2$  content,  $\pm 0.20$  volume per cent for plasma  $\text{CO}_2$  content,  $\pm 0.20$  mm. for the  $p\text{CO}_2$ , and  $\pm 0.003$  for  $\text{pH}_s$ .

the first. The difference in  $pH_s$  was 0.001, 0.003, 0.005, and 0.009; the difference expected from the change in  $pCO_2$  (in the same order) was 0.012, 0.013, 0.011, and 0.030; and the difference expected from the change in bicarbonate was 0.013, 0.010, 0.016, and 0.021.

In other words, in the normal, under basal conditions, while the changes in concentration of the weak acids or of their salts are confined within narrow limits, the magnitude of the changes in either may be appreciable and considerably larger than that of the changes in arterial  $pH_s$ . That is, despite changes in the concentration of the weak acids or of their salts (and because each such change in the concentration either of a weak acid or of its salt is, in terms of the effect on  $pH_s$ , accompanied by an approximately equivalent and compensatory change in the other), the arterial  $pH_s$  is kept from varying, except for a very small amount, from the mean value for the subject and from that for the group.

Both the initial and compensatory changes in the concentration of the weak acids and of their salts in the blood of each of the four subjects mentioned above originate elsewhere than in the blood. First, there was an appreciable difference in the over-all heights of the  $CO_2$  dissociation curves on the two occasions; *i.e.*, the blood gained or lost base or acid (other than carbonic) from or to the tissues, or the organs, or both. Secondly, the concentration of carbonic acid changed in the same direction as that of bicarbonate; *i.e.*,  $CO_2$  was gained by or lost from the blood. In this regard it will be shown in a subsequent communication that, when there is a change in the amount of  $CO_2$  to be eliminated, the tissues effect the circulatory adjustments and changes in external respiratory activity necessary to alter the elimination of this metabolite, by effecting changes in the arterial blood. The blood is but a part of the larger system, an intermediary among the several tissues and organs, serving its function by reflecting changes in various parts of the organism under the dominance of the tissues.

It seems reasonable therefore to accept the findings of these experiments as indicating the status of the several tissues. That is, in the normal subject under basal conditions, despite changes in the concentration of the weak acids and their salts, the tissue  $pH$  is kept from varying except for a very small amount.

These findings are consistent with the concept that the maintenance of the tissue  $pH$  is a primary function of the organism. They indicate that it is of greater importance in the body economy to minimize change in  $pH$  than to maintain a given concentration of the weak acids or of their salts or to minimize changes in these concentrations, and suggest that one function served by this maintenance of  $pH$  within so narrow a range is to keep it, the tissue  $pH$ , at a level which is optimum for the physicochemical processes characterizing the resting state.

## SUMMARY

The pH<sub>i</sub> of the arterial blood of each of eighteen young men in the basal state was determined by the gasometric method.

The sensitivity of the method was tested and, in terms of pH<sub>i</sub>, was found to be  $\pm 0.003$ .

It is our impression from our work thus far that under basal conditions the fluctuations in arterial pH<sub>i</sub> of each normal individual are limited to changes in the third place and that the variation among such individuals, in terms of the means of these individual ranges, is limited to differences in the third place.

It is concluded that, in the normal under basal conditions, the pH of the several tissues as well as that of the arterial blood is kept from varying except for a very small amount, despite changes in the concentration of the weak acids and of their salts.

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# EFFECT OF DIPHOSPHOPYRIDINE NUCLEOTIDE ON THE RATE OF OXIDATION OF BETAINIC ALDEHYDE\*

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Evidence has been presented indicating that betainic aldehyde is oxidized to betaine by rat liver (1). It was found in the present study that the rate of oxidation of betainic aldehyde by preparations of certain rat tissues was increased by the addition of diphosphopyridine nucleotide.

## EXPERIMENTAL

The oxidation of betainic aldehyde by washed preparations (2) of rat liver and other tissues and the effect of diphosphopyridine nucleotide on the oxidation were measured manometrically at 38°. The betainic aldehyde was used in the form of the chloride, which was prepared from chloroacetal (3). Nicotinamide was added to the experimental mixtures in order to delay the inactivation of the added nucleotide (4, 5).

The data in Fig. 1 show that diphosphopyridine nucleotide in the presence of nicotinamide increased the rate of oxidation of betainic aldehyde and of choline. The rates of oxidation of the aldehyde and of choline at pH 7.8 were not affected by 0.1 M nicotinamide in the absence of the nucleotide, or by the nucleotide in the absence of the amide. At pH 6.7 the oxidation of choline in the presence of nicotinamide was not appreciably affected by the addition of the nucleotide. Choline is oxidized to betainic aldehyde at pH 6.7, and to betaine at pH 7.8 (1). Therefore, the effect of the nucleotide on the oxidation of choline at pH 7.8 was due presumably to its effect on the oxidation of the aldehyde. Equal amounts of triphosphopyridine nucleotide, in the form of a preparation of purity 0.1, had no effect in the oxidation of the aldehyde or choline.

The effect of the nucleotide on the rate of oxidation of the aldehyde could be detected when the concentration of the former was 0.05 mg. per 2 ml. but not when the concentration was 0.005 mg. per 2 ml. Concentrations of nicotinamide greater than 0.1 M had an inhibitory effect on the rate of oxidation; however, the increased rate produced by the nucleotide was not appreciably affected by concentrations up to 0.22 M. The effect of the

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nucleotide was obtained occasionally when the concentration of amide was 0.04 M.

As indicated by the data in Fig. 2, the optimum pH for the oxidation of the aldehyde by the liver preparation was about 8.0. The final oxygen uptake in the pH range 7.3 to 8.3 was usually somewhat more than the amount corresponding to the utilization of 1 atom of oxygen per mole of aldehyde. The final oxygen uptake obtained with choline at pH 7.8 was

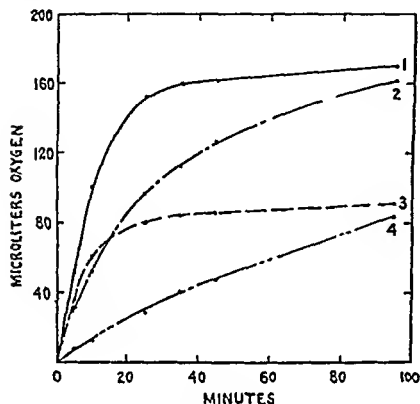


FIG 1

FIG 1 Effect of diphosphopyridine nucleotide on the oxidation of betaine aldehyde and choline. Each curve represents the oxygen uptake of an experimental mixture containing substrate minus the uptake of an appropriate control. The experimental mixtures were made up to a final volume of 2 ml. with 0.05 M phosphate buffer, pH 7.8, and contained 0.5 ml. of liver preparation, 0.0064 mM of choline or 0.0065 mM of betaine aldehyde, sufficient nicotinamide to give a final concentration of 0.1 M, and, as indicated, 0.5 mg. of diphosphopyridine nucleotide (in the form of a preparation of purity 0.5). Curve 1, choline plus nucleotide. Curve 2, choline. Curve 3, betaine aldehyde plus nucleotide. Curve 4, betaine aldehyde.

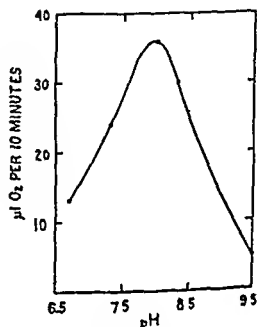


FIG 2

FIG 2 Effect of pH on the rate of oxidation of betaine aldehyde. The experimental mixtures were the same as those indicated for Curve 3, Fig. 1.

also somewhat more than was required for oxidation to betaine. This extra oxygen uptake was presumably due to a slow oxidation of betaine, since the liver preparation oxidized betaine slowly. The ratio of the rate of oxidation of betaine to that of the aldehyde was 0.05.

No evidence for dismutation of betaine aldehyde was obtained. Dismutation was tested for anaerobically (6) and as follows: Five samples of the mixture of liver, amide, and nucleotide, in the concentrations indicated for Curve 3, Fig. 1, were treated with nitrogen in order to remove oxygen.

To Sample 1 was added 0.013 mm of betaine aldehyde, to Sample 2, 0.0065 mm of choline. The samples were then kept under anaerobic conditions for a period of time sufficient to permit the aerobic oxidation of the same amount of aldehyde in a parallel experiment. The mixtures were then acidified to pH 6.7, quickly aerated, 0.0065 mm of choline added to Sample 3, and 0.013 mm of aldehyde to Sample 4. Sample 5 served as a control. The oxygen uptakes of all samples were then measured. The rate and extent of oxidation of the substrates in Samples 1 and 4 and in Samples 2 and 3 were the same, the oxidation of the two pairs being different. If dismutation of the aldehyde in Sample 1 to choline and betaine occurred, the rate and extent of the aerobic oxidation would have been the same as in Samples 2 and 3. Since the rate and extent of aerobic oxidation of the aldehyde previously incubated anaerobically (Sample 1) was the same as that of the aldehyde subjected to aerobic oxidation alone (Sample 4), dismutation did not occur. Similar experiments in which the rates of aerobic oxidation were compared at pH 7.8 also did not indicate dismutation.

Betaine aldehyde was also oxidized by preparations of kidney, brain, and muscle, the activities of these tissues compared to liver being 0.33, 0.13, and 0.10 respectively. The relative activities were compared in the presence of the nicotinamide and diphosphopyridine nucleotide. The nucleotide had the same effect on the rate of oxidation of the aldehyde by these tissues as in the case of liver.

#### SUMMARY

The rate of oxidation of betaine aldehyde by preparations of rat liver, kidney, brain, and muscle was increased by diphosphopyridine nucleotide. Triphosphopyridine nucleotide did not affect the oxidation. Dismutation of the aldehyde by the liver preparation did not occur.

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# THE INTERACTION OF THE BLOOD PROTEINS OF THE RAT WITH DIETARY NITROGEN\*

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When small amounts of amino acids or  $\text{NH}_3$  containing heavy nitrogen were added as a marker to the normal diet of rats, nitrogen of dietary origin was found to be rapidly incorporated into the proteins of animal tissues. It has been shown (1-4) that the presence of  $\text{N}^{15}$  is due to the replacement of individual amino acids in the body proteins by their isotopic analogues supplied in the diet directly or formed as a result of intermolecular transfer of nitrogen during temporary liberation of amino groups. The rate of appearance of  $\text{N}^{15}$  in a protein, when an isotopic amino acid is supplied in the diet, is therefore influenced by the rate of splitting and formation of peptide bonds as well as by the rates of all the metabolic reactions involving the shift of nitrogen from one amino acid to another. The  $\text{N}^{15}$  values obtained by such procedures may, however, serve as a means of comparing the "chemical activity" or rate of "regeneration" of the body proteins of specific organs or tissues. The chemical activity of muscle and skin proteins is less than that of the proteins of internal organs (1-3), and it seems probable that the proteins of liver parenchyma cells react much more rapidly than those of the connective tissue of the same organ. The values earlier reported for whole organs therefore represent the average activity of a highly complex mixture. An investigation of the separate cell systems constituting an internal organ is not at present entirely feasible, and it is difficult to fractionate organ proteins of the small animals to which one is restricted by the scarcity of isotopes.

The blood proteins offer a suitable material for such investigation; in contrast to organs, plasma may be more readily sampled and more conveniently fractionated into component proteins. These and the erythrocyte proteins have been studied with the aid of heavy nitrogen.

*Plasma Proteins*—The regeneration of plasma proteins was investigated

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by supplementing for 3 days the casein-containing stock diet of normal adult rats with amino acids marked with  $N^{15}$ .<sup>1</sup> In the case of tyrosine the supplement, 23 mg. of N per rat per day, was administered for a longer period, namely 10 days. In all other respects the experimental conditions were uniform.

In this investigation of blood proteins, compounds have been administered with isotope concentrations ranging from 4.5 to 10.5 atom per cent  $N^{15}$  excess. The isotope concentrations observed in the proteins and individual amino acids isolated from the animals have been recorded on a uniform basis by the procedure (2) of calculating all values as for 100 per cent  $N^{15}$  in the nitrogen administered. This form of presentation has the advantage that it shows directly what percentage of the nitrogen of the biological reaction product is derived from the administered nitrogen. If a serum protein fraction is reported by this convention to contain "2.00 atom per cent marked nitrogen," 2 per cent of the protein nitrogen was derived from the labeled glycine administered.

In Table I are given the isotope concentrations of the plasma proteins of these animals and for comparison the corresponding data, previously reported, for several internal organs. The concentration of marked nitrogen in the plasma protein is slightly lower than that of the liver in the experiment with *dl*-tyrosine but somewhat higher than that of the internal organs in the other experiments recorded in Table I. The rate of "regeneration" of plasma proteins is thus approximately equal to that of the average proteins of liver, kidney, or the intestinal tract.

The plasma samples obtained from animals given either *d*- or *l*-leucine or glycine were fractionated by precipitation with increasing concentrations of sodium sulfate.<sup>2</sup> There is little difference between the isotopic levels in the four fractions of each experiment. The results indicate that the various plasma proteins of a normal animal are involved at similar rates in the chemical reactions responsible for the introduction of dietary nitrogen and may be considered to have about the same "chemical activity" as the average proteins of the internal organs investigated.

*Source of Plasma Protein*—Whipple and collaborators (5), as a result of experiments on the replenishment of plasma proteins lost by plasmapheresis, consider that a dynamic equilibrium exists in the body whereby the proteins of the plasma, liver, and other tissues are constantly exchange-

<sup>1</sup> The observations reported here were made on samples of blood obtained during detailed investigations of the intermediary metabolism of the isotopic amino acids *dl*-tyrosine, *l*-leucine, *d*-leucine, and glycine. The methods of synthesis and analysis and the results obtained on feeding these compounds have been reported elsewhere (1-4).

<sup>2</sup> See the following paper for experimental details.

ing. The results obtained with normal animals in nitrogen equilibrium require a similar interpretation. They demonstrate the continuous chemical interactions of serum proteins with body proteins and diet. These reactions occur rapidly and presumably while the serum proteins are in contact with organ cells.

Though the origin of plasma proteins is not fully understood, the liver is most frequently considered to be the site of their formation (5). Our data are not in disagreement with this theory, since, as was pointed out above, the values reported for the whole organ represent the average of a mixture of proteins of varying activities. A fractionation of the liver

TABLE I

*Concentration of Marked Nitrogen in Internal Organs and Plasma Protein Fractions of Rats Given Isotopic Amino Acids*

The values are calculated for an isotope content of 100 atom per cent  $N^{15}$  in the amino acid administered

Protein fraction	Compound administered			
	<i>l</i> Leucine	<i>d</i> Leucine	Glycine	<i>dl</i> -Tyrosine
	atom per cent	atom per cent	atom per cent	atom per cent
Total plasma protein	1 65	1 15	1 77	1 45
Fibrinogen	1 79	1 06	2 35	
Euglobulin	1 51	0 89	1 82	
Pseudoglobulin	1 78	1 20	1 76	
Albumin	1 72	1 04	1 67	
Liver protein	0 93	0 89	1 40	1 95
Kidney "	1 36	0 97		
Intestinal tract protein	1 48	0 76	0 97	
Erythrocytes	0 29	0 28	0 44	0 45
Hemin	0 14			
Probable error of calculated values	±0 04	±0 04	±0 06	±0 15

might well show the presence of some proteins having isotope concentrations higher than those of the corresponding plasma.

*Proteins of Erythrocytes*—In contrast to the proteins of the plasma and organs, those of the red blood corpuscles were found to have low concentrations of isotope. Of the several constituents of hemoglobin, only the heme and globin can be investigated with heavy nitrogen. However, it is known from the investigations of Hahn, Bale, Lawrence, and Whipple with radioactive iron (6) that the iron of hemoglobin is replaced very slowly in normal animals. Both the red cells and their porphyrin were studied in the experiment in which *l*-leucine was fed. The hemin contained an even smaller concentration of  $N^{15}$  than the red cells. Hemoglobin, the principal



protein within the red cell, therefore has a low "chemical activity" when compared to the plasma proteins. This suggests that the protein of hemoglobin, in the circulation, is involved in only a slow cycle of synthesis and destruction. This idea is in accord with the evidence obtained with radioactive iron, which indicates that the lifetime of the red cell is relatively long (*cf.* also (7)).

#### SUMMARY

1. By administration of isotopic amino acids to rats, plasma proteins have been shown to take part in metabolic reactions involving the incorporation of dietary nitrogen.

2. The rate of this process in the plasma proteins when compared with that in the kidney, liver, and intestinal tract of the same animals is approximately the same, and all fractions of the plasma protein, fibrinogen, euglobulin, pseudoglobulin, and albumin participate to about an equal extent.

3. Investigation of the erythrocytes under the same conditions shows that both the protein and the porphyrin of hemoglobin have a lower "chemical activity" than the plasma proteins.

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# THE INTERACTION OF ANTIBODY PROTEIN WITH DIETARY NITROGEN IN ACTIVELY IMMUNIZED ANIMALS\*

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When an animal is given antigen parenterally, new substances known as antibodies may appear in the plasma. There is now ample evidence (1) that these antibodies are globulins which differ from those normally present chiefly in a specific reactivity with the antigen used. The actual formation of antibodies must therefore involve some change, as yet only vaguely defined, in the structure of the globulin molecule. According to current theories (2) appearance of antibodies is dependent upon the presence of antigen or its degradation products at the site of globulin formation. The antigen must therefore be regarded as having more or less direct influence on the formation of these specific proteins. Classical experimental procedures do not reveal with certainty whether antibodies, once formed, circulate unchanged prior to complete degradation, or whether they take part in the replacement reactions characteristic of other plasma (3) and organ proteins (4). The uptake of dietary nitrogen which accompanies protein synthesis through amino acid replacement and nitrogen transfer among individual amino acids has been employed (3) as a criterion of protein regeneration.

Precipitation of antibody from serum or plasma by addition of homologous antigen furnishes an ideal method for the separation of this characteristic protein, and its quantitative estimation is also simple and accurate (5). The chemical reactions in which antibody is involved have been investigated by administration of isotopic amino acids to immunized animals. The isotope analysis of the antibody, isolated as the antigen-antibody complex, showed that antibody, even at the time when it was decreasing in total amount, was involved in chemical interactions with other nitrogenous compounds.

*Interaction of Antibody Protein with Dietary Glycine in Rat—7 days*

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after the final injection of a rat with hemocyanin (Experiment 1) isotopic glycine was administered for 3 days by admixture with the stock diet. The antigen-antibody complex isolated from the blood plasma at the end of the experimental period by precipitation with hemocyanin contained much  $N^{15}$  (Table I). About 1.7 per cent of the nitrogen of the antibody protein and 2.0 to 2.3 per cent of the nitrogen of the other serum proteins were derived from the dietary glycine in 3 days.

*Interaction of Antibody Protein with Dietary Tyrosine and Glycine in Rabbit*—Since rats do not form precipitins readily, the subsequent experiments were carried out with rabbits. Pneumococcus Type III was employed for immunization. As the nitrogen-free specific carbohydrate from this microorganism was used for precipitation of the antibody, the nitrogen of the insoluble antigen-antibody complex was derived exclusively

TABLE I

*Concentration of Marked Nitrogen in Plasma Proteins of Immunized Rat Given Isotopic Glycine*

The values are calculated for an isotope content of 100 atom per cent  $N^{15}$  in the glycine administered. The probable error of these values is  $\pm 0.07$ .

Total protein	Antibody	Fibrinogen	Euglobulin	Pseudoglobulin	Albumin
<i>atom per cent</i>	<i>atom per cent</i>	<i>atom per cent</i>	<i>atom per cent</i>	<i>atom per cent</i>	<i>atom per cent</i>
2.30	1.69	2.30	Not determined	2.28	2.18

from the antibody. A preliminary experiment (Experiment 2) was carried out by administering isotopic tyrosine by stomach tube to a rabbit 7 days after immunization was completed. The  $N^{15}$  content in antibody, serum protein, and non-protein nitrogen isolated from blood samples obtained at intervals during an 8 day period following isotope administration is given in Table II.

Both antibody and serum proteins acquired about the same low concentration of isotope. These values involve an appreciable error, but are far above the limit of error of the analysis. The isotope concentration in the non-protein nitrogen fraction is high.

The "activity" of the serum proteins in this experiment is very low when compared with that of other experiments in which tyrosine or glycine was given to rats (6, 7). The protein of the rabbit took up only a small fraction of the administered tyrosine nitrogen. As was found later, this was due to the abnormal method of amino acid administration. If the isotopic compound is given, as in this experiment, in one large dose by stomach tube, its utilization appears to be less efficient than when it is administered

physiologically as a minor component of the diet. The latter procedure is therefore preferred. Both preliminary experiments in the rat and rabbit, however, show that antibody protein is involved in reactions resulting in the introduction of dietary nitrogen.

The low values obtained in the preceding experiment did not permit an extensive study of antibody or plasma protein regeneration. An immune rabbit (Experiment 3) was therefore given isotopic glycine containing 4.5 atom per cent  $N^{15}$  excess, by admixture with the stock diet, for 3 days, beginning 10 days after the final injection of pneumococci. This interval was chosen because at this point the amount of circulating antibody began to decrease. Subsequent antibody levels are given in the lower portion of Fig. 1. Following this period the animal was kept on the same diet

TABLE II  
*Concentration of Marked Nitrogen in Serum Protein and Antibody of Immunized Rabbit Given Isotopic Tyrosine*

The values are calculated for an isotopic content of 100 atom per cent  $N^{15}$  in the tyrosine administered. The probable error of these values is  $\pm 0.03$ .

Hrs. after feeding	Antibody content of serum	$N^{15}$ concentration in			Hrs. after feeding	Antibody content of serum	$N^{15}$ concentration in		
		Antibody	Remain- ing serum protein	Non- protein N			Antibody	Remain- ing serum protein	Non- protein N
	mg N per cc.	atom per cent	atom per cent	atom per cent		mg N per cc.	atom per cent	atom per cent	atom per cent
3.0	2.48	0 10	0.12	7 2	33.5	2.26	0.24	0.31	
5.5	2.40	0 20	0.22	5 3	49.5	2.28	0 23	0.31	0 57
9.2	2 29	0 23	0.28	5 5	128	2 06		0 19	0 27
15.5	2 45	0 23	0.29	2 3	176		0 25	0 20	
26.5	2 39	0 25	0.29	1 1	200		0 15	0 18	

without the addition of glycine. During and after glycine feeding the total amount of antibody and the isotope content of the antibody and of the rest of the serum proteins were determined in samples of blood obtained at frequent intervals over a period of 20 days. These values are recorded in the upper portion of Fig. 1. Immediately after the addition of isotopic glycine to the diet, the isotope content in the nitrogen of antibody and serum protein rose rapidly. After 3 days about 1.5 per cent of the nitrogen of each of the proteins is marked nitrogen originating from the dietary glycine. After the isotopic glycine in the diet was stopped, the isotope content in both antibody and non-antibody proteins decreased. There is little doubt that the same type of chemical reaction was responsible for both the increase and the decrease of isotope concentration. The rate of nitrogen replacement in the antibody, which almost parallels that of the

remaining serum proteins, fails to distinguish this protein from others normally present.

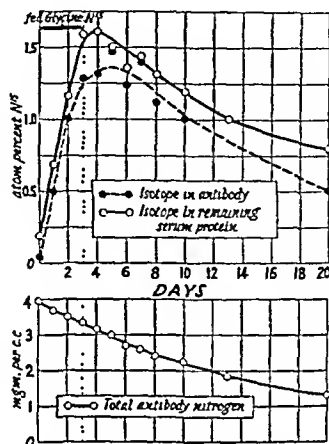


FIG. 1. Concentration of marked nitrogen in serum protein and antibody of immunized rabbit during and after the feeding of isotopic glycine (calculated for an isotopic content of 100 atom per cent in the compound administered).

TABLE III

*Concentration of Marked Nitrogen in Amino Acids from Fractions of Serum of Immunized Rabbit Given Isotopic Glycine*

The values are calculated for an isotope content of 100 atom per cent N<sup>15</sup> in the glycine administered. The probable error of these values is  $\pm 0.06$ .

Compound	Antibody	Globulin A*	Euglobulin	Pseudoglobulin	Albumin
	atom per cent	atom per cent	atom per cent	atom per cent	atom per cent
Total protein	0.89	1.84		2.38	1.52
Glutamic acid	0.77	1.68	1.38	2.88	2.34
Aspartic "	0.49	1.34			2.28
Arginine		0.64	0.48	1.82	1.34
Insoluble Cu salts of monoaminomonocarboxylic acids	1.20				
Glycine	6.82				

\* Precipitated by 25 per cent saturation with Na<sub>2</sub>SO<sub>4</sub>.

*Distribution of Dietary Nitrogen in Serum Proteins and Antibody*—Since the isotope concentration in the whole protein cannot alone reveal the nature of the reactions responsible for the introduction of isotope, an

attempt was made to investigate the reactions in which antibody and other serum proteins are involved by isolation of individual amino acids from these proteins. In Experiment 4 an immune rabbit was given isotopic glycine under the same conditions as in Experiment 3, after which the animal was exsanguinated by heart puncture. The blood plasma was separated into antibody and other plasma protein fractions and amino acids were isolated from each fraction by suitable procedures. The isotope concentrations of the individual amino acids are given in Table III.

The results are similar to those found in a study of the proteins of organs obtained from rats that were given isotopic amino acids (4, 6; 7). The glycine isolated from the antibody protein had a very high isotope content, indicating that dietary glycine had replaced some of the glycine originally present in the protein. The other amino acids had lower but significant isotope concentrations in all the protein fractions. The reactions indicated by the presence and relative concentration of isotope in the various amino acids of the plasma proteins are therefore essentially the same as those deduced for the organ proteins. They require a continuous opening and closing of peptide linkages for the introduction of new amino acids and a shift of  $\alpha$ -amino nitrogen. A discussion of these reactions and their metabolic significance has already been given (4). The present studies show that antibodies are involved in the same cycle of metabolic reactions as the other proteins of serum and of the organs.

#### EXPERIMENTAL

*Experiment 1*—A rat weighing 350 gm. received thirty-two intraperitoneal injections of alum-precipitated *Limulus* hemocyanin averaging about 2 mg. of protein per injection. During the whole experimental procedure the animal was kept on the casein-containing stock diet (6). 7 days after the last injection of hemocyanin, isotopic glycine (4.5 atom per cent  $N^{15}$  excess) corresponding to 37 mg. of nitrogen per day was fed for 3 days. At the end of this period the animal was killed by heart puncture. 0.78 mg. of specific precipitate nitrogen was obtained by addition of 2.5 mg. of hemocyanin nitrogen to the 4.5 cc. of plasma available. The supernatant plasma from the specific precipitate was fractionated as described below by treatment with varying concentrations of sodium sulfate.

The nitrogen of the antigen-antibody complex contained 0.037 atom per cent  $N^{15}$  excess. The values reported by Malkiel and Boyd (8), who studied rabbit antihemocyanin, suggest that this precipitate might consist of about one-third hemocyanin and two-thirds antibody. As the hemocyanin in our experiment did not contain marked nitrogen, the  $N^{15}$  value in the antibody nitrogen was calculated from the observed value after both

this dilution and a second one, necessitated by the addition of a known quantity of normal nitrogen, as described below, in order to bring the total quantity up to 1 mg., were taken into account. The calculated value for  $N^{15}$  in the antibody was 0.07 atom per cent  $N^{15}$  excess.

*Experiment 2*—Rabbit A-11, weighing about 3 kilos, which had received several earlier courses of intravenous injections of formalinized Type III pneumococci, was again given a short course of injections. On the 6th day after the final injection a sample of the animal's serum contained 2.68 mg. of antibody nitrogen per cc. On the following day the animal was given 1.5 gm. of *dl*-tyrosine (containing 10.5 atom per cent  $N^{15}$  excess) as the sodium salt in 17 cc. of water by stomach tube. Samples of blood were taken at intervals; antibody was quantitatively precipitated with a slight excess of specific polysaccharide (5) and  $N^{15}$  was determined in the precipitate, in the protein of the antibody-free serum, and in the non-protein nitrogen. The results are given in Table II.

*Experiment 3*—Rabbit A-36 which, like Rabbit A-11, had received a number of courses of Type III pneumococcus vaccine injections, was kept in a metabolism cage and received daily 50 gm. of a mixture of 2 parts of ground whole wheat and 1 part of ground alfalfa. This was administered as a wet mush in a cup specially designed to prevent spilling. The animal consumed all of its diet. Small daily samples of blood were obtained and the amount of antibody nitrogen per cc. of serum was determined daily.

10 days after the last antigen injection, when the total amount of circulating antibody was decreasing, there was added to the diet 1.5 gm. of glycine (4.5 atom per cent  $N^{15}$  excess) per day for 3 days. Following this period the animal was kept on the same diet without the addition of glycine. During and after glycine feeding, blood samples were withdrawn daily and fractionated. The results of isotope analysis, expressed as in Table I, are given in Fig. 1.

*Experiment 4*—A rabbit weighing 2.5 kilos was immunized with Type III pneumococcus as in Experiment 2. 7 days after the last injection, there were added to the diet 1.75 gm. of glycine (4.93 atom per cent  $N^{15}$  excess) per day for 3 days. At the end of this period the animal was bled from the heart. Antibody was precipitated from the serum with specific polysaccharide. The washed precipitate contained 246 mg. of nitrogen, corresponding to about 1.5 gm. of antibody protein. The supernatant fluid was fractionated with sodium sulfate into the various protein fractions as described below. Each fraction was hydrolyzed and a number of pure amino acids (glutamic acid, aspartic acid, arginine, and glycine) were isolated. The results of the isotope analysis are given in Table III.

*Chemical Procedures*—The isotopic amino acids, *dl*-tyrosine and glycine, were prepared by methods previously described (9). The fractionation

of serum or plasma was carried out by precipitation with sodium sulfate solution saturated at 35–38°. In general, an equal volume of water was added to the centrifuged serum, followed by sufficient saturated  $\text{Na}_2\text{SO}_4$  solution to bring the salt concentration to one-quarter saturation. Any precipitate was centrifuged off and arbitrarily considered fibrinogen or globulin A. The supernatant was brought to one-third saturation with  $\text{Na}_2\text{SO}_4$  and the precipitate designated euglobulin. Subsequent precipitation at one-half saturation gave "pseudoglobulin" and full saturation yielded albumin which was usually filtered off in a warm place and redissolved in water. In one or two instances fibrinogen was obtained from plasma by warming to 56° and stirring. Non-protein nitrogen was removed from each protein solution or sample of supernatant serum by precipitation of the protein with trichloroacetic acid. The filtered and washed precipitate was then dissolved in dilute alkali to facilitate sampling. For analysis of non-protein nitrogen, the trichloroacetic acid filtrates were extracted with ether and the aqueous layer employed for estimation of total nitrogen and  $\text{N}^{15}$  content.

The determination of antibody nitrogen in serum was carried out as previously indicated (5) by addition of a slight excess of antigen or specific polysaccharide to an accurately measured volume of serum. The specific precipitate was washed twice in the cold with saline and analyzed for nitrogen by a modification of the micro-Kjeldahl method. Estimations of  $\text{N}^{15}$  were carried out as usual (10) except when the amount of nitrogen was inconveniently small as in the specific precipitate from Experiment 1 and in non-protein nitrogen samples. In such cases ammonium acid phthalate was added to increase the gas volume. In reporting such values (Tables I and II) we have taken the dilution into account. The concentration of  $\text{N}^{15}$  in the non-protein nitrogen of Experiment 2 (Table II) is much greater than the corresponding serum protein value at the beginning of the experiment, and rapidly decreases. In Experiment 3 (Fig. 1), when the isotopic amino acid was administered, not by stomach tube, but in the diet over a 3 day period, the concentrations of  $\text{N}^{15}$  in the non-protein nitrogen of the serum for the first 5 days were 4.4, 4.9, 6.6, 4.2, and 4.7 per cent respectively. It is highly probable that a considerable part of the heavy nitrogen in the non-protein nitrogen was in the form of the amino acid fed. The method of administration in Experiment 3 which results in a much slower absorption than in Experiment 2 may reasonably explain the maintenance of the non-protein nitrogen at a high level of  $\text{N}^{15}$  during feeding and for 48 hours after.

In Experiment 4, for the isolation of amino acids, antibody was separated from the remaining serum proteins by adding to the diluted serum a solution of 56.4 mg. of the specific polysaccharide of Type III pneumococcus



(11), slightly more than the amount necessary to precipitate the antibody. The precipitate was centrifuged off and washed three times with 40 to 50 cc. of chilled saline. The last washing still contained heat-coagulable protein.

*Isolation of Amino Acids from Antibody and Other Serum Proteins of Experiment 4*—The specific precipitate was hydrolyzed with 20 per cent hydrochloric acid. The hydrolysate contained 246 mg. of nitrogen. It was brought to dryness *in vacuo*, and chloride ions were removed by treat-

TABLE IV

*Analysis of Amino Acids Isolated from Serum Proteins of Immunized Rabbit Given Isotopic Glycine*

The N<sup>15</sup> concentrations reported here are the observed values having a probable experimental error of  $\pm 0.003$ .

	Antibody		Globulin A*		Euglobulin		Pseudoglobulin		Albumin	
	Total N	N <sup>15</sup> concentration	Total N	N <sup>15</sup> concentration	Total N	N <sup>15</sup> concentration	Total N	N <sup>15</sup> concentration	Total N	N <sup>15</sup> concentration
	per cent	atom per cent N <sup>15</sup> excess	per cent	atom per cent N <sup>15</sup> excess	per cent	atom per cent N <sup>15</sup> excess	per cent	atom per cent N <sup>15</sup> excess	per cent	atom per cent N <sup>15</sup> excess
Glutamic acid hydrochloride; theory 7.60% N	7.40	0.038	7.45	0.084	7.65	0.069	7.72	0.144	7.60	0.117
Aspartic acid as anhydrous copper salt; theory N 7.18	7.08	0.024	7.24	0.067					7.00	0.114
Glycine as toluene-sulfonylglycine; theory N 6.11	6.20	0.341								
Arginine				0.032		0.024		0.091		0.067

\* Precipitated by 25 per cent saturation with Na<sub>2</sub>SO<sub>4</sub>.

ment with silver carbonate; the amino acids were precipitated by Neuberg's procedure (12) and liberated with hydrogen sulfide. The other protein hydrolysates were worked up without preliminary precipitation of amino acids. Arginine was precipitated as the flavianate, and the flavianate recrystallized. The product was decomposed with concentrated hydrochloric acid; the filtered arginine solution was treated with norit and used directly for isotope determination. The dicarboxylic acids were precipitated as barium salts with alcohol. Glutamic acid was isolated as the hydrochloride and aspartic acid as the copper salt. After removal of the dicarboxylic acids the hydrolysate was treated with copper carbonate, and the fraction of copper salts insoluble in water was separated. Its amount

was too small (24 mg.) for further fractionation. After removal of copper from the filtrate, glycine was precipitated with oxalatochromiate (13) and purified as toluenesulfonylglycine (m.p. 147°), as described previously (7).

The total nitrogen and isotope concentration of the amino acid samples are given in Table IV.

#### DISCUSSION

The interaction of dietary nitrogen with antibody protein has been observed in antiprotein and anticarbohydrate for varying periods of time. All the experiments were carried out after maximum antibody formation had been attained and when the total amount of circulating antibody was actually declining. During the first few days of this experiment and simultaneously with the administration of isotopic glycine, the  $N^{15}$  content of the antibody rose rapidly; this was followed by a continued decrease in  $N^{15}$  when isotopic glycine was no longer fed. The introduction of isotopic nitrogen into antibody protein under these conditions must have involved both the opening and closing of peptide bonds in protein degradation and synthesis. Similarly the removal of isotopic nitrogen from the antibody after the 4th day could only have occurred by a continuation of the same reactions responsible for the introduction of isotope. The data supplied in Fig. 1 therefore permit an estimate of the rates of formation of both antibody and serum protein. The concentration of marked nitrogen in the antibody nitrogen fell from a maximum of about 1.6 atom per cent to one-half that value in about 2 weeks. This period may be taken to represent the half lifetime of nitrogen in antibody protein and probably closely approximates the actual half lifetime of the antibody molecule. That of the average serum protein molecule is of about the same duration.

#### SUMMARY

1. By the administration of isotopic amino acids to actively immune rat and rabbits, it has been shown that antibody, like the other serum and body proteins, participates in metabolic reactions involving the uptake of dietary nitrogen.

2. The distribution of isotopic nitrogen introduced into antibody and serum protein fractions has been investigated by the isolation of amino acids. The observed isotope concentrations indicate that amino acid replacement and nitrogen transfer among individual amino acids occur in antibody and normal serum proteins, in the same manner as has been indicated in organ proteins.

3. The average rate of these reactions has been observed in antibody and normal serum proteins by following the rate of replacement of isotopic nitrogen by normal nitrogen. It was found that the half life of an antibody mole-

cule is about 2 weeks, approximately the same as that of the average serum protein.

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# BEHAVIOR OF ANTIBODY PROTEIN TOWARD DIETARY NITROGEN IN ACTIVE AND PASSIVE IMMUNITY\*

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In the preceding paper (1) it was shown that actively induced antibody protein does not differ from other plasma or body proteins with respect to the uptake of dietary nitrogen but participates in the same types of metabolic reactions and at about the same rate. In order to avoid undue complications, the period chosen for investigation followed the peak of antibody production, and observations were made when the total amount of circulating antibody was actually declining. Under these conditions, indications of continued antibody formation were obtained.

A further opportunity for the investigation of a specific protein is offered by passive immunization, in which appreciable amounts of antibody produced in one animal are injected into another. When antipneumococcus serum is employed for this purpose, quantitative separation from other proteins in the plasma may be carried out with specific polysaccharide as in the earlier experiments. It should then be possible to determine whether a protein homologous to the species of animal used, but artificially introduced, would show the same behavior as actively produced antibody or other native proteins in the body (2).

A normal rabbit was given a single large injection of Type III antipneumococcus rabbit serum. The administration of isotopic glycine by addition to the stock diet was started 2 hours before injection and continued for 48 hours. Daily estimations of the amount of circulating antibody and of the  $N^{15}$  concentration of antibody and residual serum proteins were made. As noted in Table I, the antibody protein contained a very low concentration of  $N^{15}$ , though the high values usually observed were found in the other serum proteins.

The error of analysis of  $N^{15}$  by the mass spectrograph employed was esti-

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† Died, September 11, 1941.

mated to be about  $\pm 0.03$  atom per cent  $N^{15}$  excess when calculated on the basis of 100 atom per cent for the isotopic glycine used in this experiment. The values reported in Table I for antibody nitrogen are so little greater than the error of the method<sup>1</sup> that they cannot be regarded as indicative of any uptake of isotope. It was suspected that such traces might be due to the presence of small amounts of adhering material of high isotope concentration. Since the sensitivity of the mass spectrograph is greater than that of most analytical methods, contaminants may have escaped detection by the usual micro-Kjeldahl procedure. Further purification of the antibody was therefore carried out by dissociation of the specific precipitate with 10 per cent salt solution (3) and reprecipitation by the specific poly-

TABLE I

*Concentration of Marked Nitrogen in Antibody, Serum Protein, and Non-Protein Nitrogen of Passively Immunized Rabbit Given Isotopic Glycine*

The values are calculated for an isotope content of 100 atom per cent  $N^{15}$  in the administered compound. The probable error of these values is  $\pm 0.03$ .

Period*	Antibody N	$N^{15}$ concentration		
		Antibody	Supernatant serum protein	Non-protein N
hrs.	mg. per cc.	atom per cent	atom per cent	atom per cent
2 (Before injection)	0		0.05†	5.79
2½ (After " )	1.36	0.08	0.10	
25½	0.67	0.09	0.65	
48½	0.54	0.13	1.20	
49½	0.40	0.09		
Antiserum employed			0.06†	

\* After the beginning of isotope administration.

† A sample of the whole serum was analyzed.

‡ Blood was removed after 20 cc. of isotonic saline were injected.

saccharide. The  $N^{15}$  analysis of the purified antibody and that of the non-antibody protein remaining in solution after specific precipitation of the purified antibody are reported in Table II. The specific protein was again found to contain only traces of isotope, while the soluble non-specific protein, which was present in small amounts, contained a high concentration of  $N^{15}$ .

The antibody fraction which could not be extracted from the specific precipitate was dissolved in aqueous alkali (3, 4) and this portion was also

<sup>1</sup> Though the  $N^{15}$  analyses of biological materials to which no  $N^{15}$  has been added usually fall within the experimental error of the method, samples of blood plasma frequently give slightly higher values. All values are reported as excess over natural abundance.

separated into specific and non-specific fractions. The  $N^{15}$  analyses, also shown in Table II, are similar to those of the first separation. It therefore appears quite certain that the traces of  $N^{15}$  found in the passively introduced antibody are due to the difficulty of removing small amounts of adhering non-specific protein.

*Simultaneous Active and Passive Immunity in Same Animal*—Confirmation of the failure of injected antibody to incorporate dietary nitrogen was obtained with a rabbit which had first been rendered actively immune to Type III pneumococcus and subsequently had been passively immunized by a single large injection of antipneumococcus Type I rabbit serum. Isotopic glycine was administered as before. Antibodies to each type of pneumococcus were isolated from the serum by successive precipitation

TABLE II

*Concentration of Marked Nitrogen and Total Nitrogen of Antibody after Fractionation*

The values are calculated for an isotope content of 100 atom per cent  $N^{15}$  in the administered glycine. The probable error of these values is  $\pm 0.03$ .

Fraction*	Total N of fraction	$N^{15}$ concentration
	mg.	atom per cent
Soluble in 10% NaCl		
Precipitable by S-III	0.64	0.14
Non-precipitable	0.70	0.73
Insoluble in 10% NaCl, soluble in alkali		
Precipitable by S-III	22.20	0.10
Non-precipitable	1.20	0.65

\* The serum from which the dissociated solutions were prepared contained a total of 33 mg. of antibody nitrogen based on micro-Kjeldahl analyses of the specific precipitate from 10 cc. portions. The total antibody nitrogen dissociated was 70 per cent of the amount present.

with the respective specific polysaccharide. The precipitations were performed in pairs, the order of precipitation being reversed in each pair to test for complete separation of the two antibodies. That this was accomplished may be seen from the data in Table III. The  $N^{15}$  content of each antibody is unaffected by the order of separation. The high  $N^{15}$  content found in the Type III antibody and in the other serum proteins native to the animal is in marked contrast to the negligible value in the Type I antibody protein.

Attention is drawn to the greater rate of disappearance of the passively introduced antibody, in comparison with that actively produced. It is difficult to escape the conclusion that the maintenance of the level of active antibody by its continual production *in vivo* is reflected in the observation

that, of the two antibodies circulating in the animal, only that produced by the tissues of the host contains nitrogen of dietary origin. Whether such nitrogen is introduced exclusively during total synthesis or is also taken up in conjunction with partial fragmentation or temporary opening of structural linkages of antibody protein already present cannot be decided by the experiments here reported. As stated in an earlier paper (2),

"There are two general reactions possible which might lead to amino acid replacement: (1) complete breakdown of the protein into its units followed by resynthesis or (2) only partial replacement of units. Metabolic studies with isotopes indicate

TABLE III

*Concentration of Marked Nitrogen in Type I and Type III Antibodies and in Supernatant Serum Proteins Obtained from Actively and Passively Immunized Rabbit after Administration of Isotopic Glycine*

The values are calculated for an isotope content of 100 atom per cent  $N^{15}$  in the administered compound. The probable error of these values is  $\pm 0.03$ .

Period*	Antibody to C substance		Type I antibody		Type III antibody		Supernatant serum protein, $N^{15}$ concentration
	Total N	$N^{15}$ concentration	Total N	$N^{15}$ concentration	Total N	$N^{15}$ concentration	
hrs.	mg. per cc.	atom per cent	mg. per cc.	atom per cent	mg. per cc.	atom per cent	atom per cent
0 (Before injection)	0.16				3.12	0.07	
0 (After " )	0.13		1.09	0.07	2.30†	0.10	
22½	0.12	0.40	0.68	0.03	2.59‡	0.42	0.55
			0.66§	0.09	2.70	0.43	0.54
48	0.12	0.73	0.49	0.12	2.27‡	0.86	1.06
			0.46§	0.05	2.30	0.85	1.07
168	0.07	0.62	0.18	0.10	1.52‡	0.68	

\* After the beginning of isotope administration.

† Probably too little S-III was used for the analyses.

‡ From the supernatant after removal of Type I antibody.

§ From the supernatant after removal of Type III antibody.

|| After 48 hours 56 cc. of blood were removed and a transfusion of normal blood given for survival. 5 days later this sample was taken.

only end-results but not intermediate steps of a reaction. We have no indication as to what had happened to the protein molecule in the animals. Both reactions are conceivable. The second type, replacement of units, has been shown by Bergmann and collaborators [(5)] to occur *in vitro* under the action of proteolytic enzymes on polypeptides and the occurrence *in vivo* of these reactions has been postulated."

It seems likely that the dietary nitrogen may enter at many stages of protein metabolism. Whatever the mechanisms, the present data furnish no reason for considering the chemical reactions undergone by actively produced antibody protein to be different from those of any other serum or body protein.

## EXPERIMENTAL

*Experiment 1. Passive Immunization*—A normal rabbit, L, weighing about 2.5 kilos, was kept on a diet consisting of equal parts of ground alfalfa and ground whole wheat administered as a mush. After 7 days isotopic glycine containing 10.5 atom per cent  $N^{15}$  excess was added to the diet. 2 hours later 20 cc. of blood were removed by ear vein and 23 cc. of Type III antipneumococcus rabbit serum containing 5.8 mg. of antibody N per cc., diluted with 18 cc. of saline, were injected intravenously in two portions 10 minutes apart. The antiserum had previously been run through a sterile Chamberland L2 filter and analyzed. Feeding of isotopic glycine was continued for 49 hours, when a total of 2.65 gm. of isotopic glycine was consumed. A sample of blood was removed  $25\frac{1}{2}$  hours after isotope feeding was begun. At the end of the experimental period 50 cc. of blood were removed by heart puncture. 15 minutes later an additional 45 cc. were obtained following an intervening injection of saline. All samples of serum were analyzed for antibody content with the specific polysaccharide of Type III pneumococcus (S-III) as described in the preceding paper and for  $N^{15}$  concentration in both antibody and supernatant serum protein nitrogen. The data are recorded in Table I.

Though it is possible to remove non-specific protein completely within the error of the micro-Kjeldahl method from small analytical samples of specific precipitate such as those recorded in Table I, it was felt that the rather high isotope content of these washed precipitates might be due to residues of non-specific protein large enough to be detected by the extremely sensitive mass spectrograph. In order to provide information on this point, the final serum samples were pooled, filtered through a small alkali-washed Berkefeld V candle, and precipitated with the calculated amount of S-III. The specific precipitate was washed three times with chilled saline, centrifuged again, and treated for 1 hour at  $37^{\circ}$  with 10 per cent sodium chloride solution (3). The dissociated antibody and any other globulins present in solution after centrifugation were precipitated with an equal volume of sodium sulfate solution which had been saturated at  $35-38^{\circ}$  and centrifuged off, taken up in water, and made up to 5 cc. (Solution A). The salt-insoluble portion of the specific precipitate was worked up to a smooth suspension in water, dissolved as well as possible by being made alkaline to thymolphthalein in the cold with 0.1 N NaOH (3), and centrifuged in the cold. The supernatant was neutralized (Solution B, 137 cc.). Solutions A and B were again centrifuged and analyzed for antibody and non-antibody nitrogen and isotope concentration (Table II).

*Experiment 2. Simultaneous Study of Actively Produced and Passively Injected Antibody in Same Animal*—Rabbit B-63, weighing about 2.5 kilos,



tinued synthesis of antibody protein is absent and only its disappearance is revealed.

#### SUMMARY

1. Passively injected antibody has been shown to undergo no changes involving nitrogen replacement.

2. In a rabbit already actively immune to another antigen, the absence of uptake of dietary nitrogen by the passive antibody is in pronounced contrast to the appearance of marked nitrogen in the active antibody.

3. The incorporation of dietary nitrogen into actively engendered antibody appears to be ascribable to a specific alteration of cellular function induced by the presence of homologous antigen.

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## LETTERS TO THE EDITORS

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### A NEW COLOR TEST FOR TRYPTOPHANE IN PROTEIN HYDROLYSATES

*Sirs:*

The procedure to be described consists in adaptation of the Jolles reaction for indican in such a way that the reaction is given by tryptophane. Jolles<sup>1</sup> introduced alcoholic thymol for the detection of indican in urine and later<sup>2</sup> developed the reaction into a quantitative colorimetric procedure. Sharlit<sup>3</sup> subsequently modified the method, improving its specificity and sensitivity. Tryptophane itself gives only a faint color reaction with this test, a fact reported by Sharlit which we have fully confirmed. We have found, however, that, if tryptophane is deaminized by dilute acetic acid and sodium nitrite prior to treatment with a slight modification of the Sharlit procedure, a deep red color is obtained, the intensity of which is proportional to the quantity of tryptophane present.

Since this color reaction is not specific for tryptophane, being given by various indole compounds, it is of limited value when other indole compounds are present. In the case of proteins and protein hydrolysates, however, in which tryptophane is the only indole compound present, the test is particularly useful. We have tested all other known amino acids and humin-free sulfuric acid hydrolysates of casein and have in no instance observed the development of red color, the solution in each case remaining a pale green hue similar to that of the reagent blank.

The quantitative application of this procedure to protein analysis was carried out as follows: The protein sample was hydrolyzed by being boiled under a reflux for 20 to 22 hours with 5 ml. of 20 per cent NaOH per gm. of sample. The hydrolysate was neutralized to pH 7 with glacial acetic acid. Silica (from slight disintegration of the glass vessel) was filtered off and the volume of the filtrate adjusted, so that 2 ml. contained approximately 1 mg. of tryptophane. A 2 ml. sample of filtrate in a colorimeter tube marked at 5 ml. is treated with 0.3 ml. of sodium nitrite and 0.1 ml. of 10 per cent acetic acid and the mixture is shaken intermittently for 10 minutes. There are then added in succession 0.3 ml. of potassium persulfate (1 per cent), 0.5 ml. of thymol (1 per cent in 95 per cent ethanol), and 5 ml. of a mixture of 3 parts of 40 per cent trichloroacetic acid and 2 parts of concentrated hydrochloric acid, with thorough mixing after each addition. The tube is immediately placed in a boiling water bath for 5

<sup>1</sup> Jolles, A., *Z. physiol. Chem.*, **84**, 310 (1913).

<sup>2</sup> Jolles, A., *Z. physiol. Chem.*, **94**, 79 (1915).

<sup>3</sup> Sharlit, H., *J. Biol. Chem.*, **99**, 537 (1932-33).

minutes and is then cooled in an ice bath for 5 minutes. The sample will then have separated into two layers—an almost colorless aqueous layer above and a red layer of ethyl trichloroacetate below. All but approximately 0.3 ml. of the upper aqueous layer is then removed by a capillary pipette and the colored layer is diluted to the 5 ml. mark with glacial acetic acid. The resulting color is read in a photoelectric colorimeter (Klett-Summerson, with a No. S-54 filter) and compared with that of a similarly treated tryptophane standard.

*Tryptophane Assays of Protein Hydrolysates*

Protein or hydrolysate	Data on original samples			Recovery of added tryptophane			
	Total N	Tryptophane		Tryptophane in protein sample	Tryptophane added	Total tryptophane found	Per cent added tryptophane recovered
	mg.	mg.	per cent	mg.	mg.	mg.	
Lactalbumin (Harris)	7.08	1.24	2.80				
Casein (Harris)	8.50	0.97	1.83				
" (Sheffield, crude)	8.20	1.21	2.35	0.47	0.36	0.81	94.5
" ( " " deaminized)	5.58	0.89	2.54	0.69	0.21	0.90	100.0
Gelatin (Knox)	11.12	0.0	0.0	0.0	0.72	0.68	95.0
Amigen*	8.28	1.16	2.24				
Casein (Sheffield, crude, sulfuric acid-hydrolyzed)	15.30	0.0	0.0				

\* An enzymatic casein hydrolysate prepared by Mead Johnson and Company.

The tryptophane content of various proteins corrected for moisture and ash content tested by this technique and the recovery of tryptophane added to protein hydrolysates are shown in the accompanying table. The agreement between duplicate determinations is within less than 2 per cent.

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## THE IN VITRO FORMATION OF THYROXINE AND DIIODOTYROSINE BY THYROID TISSUE

Sirs:

Radioactive iodine ( $I^{131}$ ) has been used to demonstrate the conversion of iodide to thyroxine and diiodotyrosine by surviving slices of thyroid gland obtained from the rat, dog, and sheep. Varying quantities of thyroid tissue were placed in a bicarbonate-Ringer's solution containing approximately 0.1  $\gamma$  of  $I^{127}$  per cc. Tracer amounts of  $I^{131}$  were added to this solution. Such amounts of  $I^{131}$  contain atoms numerous enough for detection by their radioactivity but too few for chemical measurement. By use of such amounts of the labeled iodine it is possible to tag the iodide present in the tissue and bath without altering measurably their iodine content.

$I^{131}$  was added as iodide. At the end of 3 hours, the distribution of  $I^{131}$  among iodide, diiodotyrosine, and thyroxine in the tissue plus its surrounding medium was 1:6:1. In 3 hours as much as 12 per cent of the  $I^{131}$  added to the bath was found as thyroxine and as much as 70 per cent as diiodotyrosine. These compounds were determined by a modification of the procedure described elsewhere.<sup>1</sup> The presence of thyroxine  $I^{131}$  and diiodotyrosine  $I^{131}$  was verified by their repeated crystallizations to constant specific activity in the presence of added non-radioactive thyroxine and diiodotyrosine as carriers. Non-radioactive diiodotyrosine was added to the radioactive thyroxine before each crystallization of the latter. This served to wash out by dilution any radioactive diiodotyrosine that might have been present as a contaminant of the radiothyroxine. In a similar manner contaminating radiothyroxine was washed away from the radio-diiodotyrosine during each of the latter's recrystallizations.

The ability to convert iodide to thyroxine and diiodotyrosine was greatly reduced when thyroid slices were minced and was almost completely lost when they were homogenized.

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<sup>1</sup> Perlman, I., Morton, M. E., and Chaikoff, I. L., *J. Biol. Chem.*, 139, 449 (1941).



## EFFECT OF *p*-AMINOBENZOIC ACID ON THE MICRO-BIOLOGICAL ASSAY FOR NICOTINIC ACID

Sirs:

In our hands, the treatment of acid hydrolysates of casein with activated charcoal for the removal of traces of nicotinic acid<sup>1</sup> has always resulted in preparations which do not permit as great acid production by *Lactobacillus arabinosus* 17-5 as do hydrolysates which are not treated with charcoal.

In a typical experiment four aliquots of one lot of an acid hydrolysate of casein containing 100 mg. of dry matter per ml. were treated with 10 mg. per ml. of darco G-60, norit A, nuchar, and carbex E charcoals. The maximum amounts of 0.1 N acid produced in 10 ml. of media based on these charcoal-treated hydrolysates were respectively 3.9, 8.5, 6.9, and 7.8 ml., as compared to 10.5 ml. in a medium based on untreated hydrolysate. Furthermore, the curves of the response of the organism to increasing amounts of nicotinic acid in media prepared with charcoal-treated hydrolysates did not parallel the curves obtained in media based on untreated hydrolysates. If assays were performed on crude extracts of various materials with charcoal-treated caseins used in the assay medium, the results obtained at various levels did not agree, but increased as increasing amounts of the crude extracts were added to the assay tubes.

Elution of the charcoal which had been used in treating a casein hydrolysate with hot water at neutral or alkaline pH and addition of the eluate to the charcoal-treated hydrolysate restored the ability of the hydrolysate to support maximum growth.

Addition of any one of a number of supposedly pure compounds to a medium based on a hydrolysate which had been treated with 20 mg. per ml. of darco G-60 resulted in as good acid production as was obtained in a medium based on the untreated hydrolysate. The compounds and the minimum amounts of each required to support maximum growth were pimelic acid 50  $\gamma$ , inositol 100  $\gamma$ , choline hydrochloride 50  $\gamma$ , thymus nucleic acid 10  $\gamma$ , adenosine 50  $\gamma$ , xanthine 50  $\gamma$ , one brand of arginine monohydrochloride 1 mg. (another brand of arginine was without effect), and *p*-aminobenzoic acid 0.001  $\gamma$ . It will be noted that *p*-aminobenzoic acid is 10,000 times as active as the next most active compound. This fact suggests that *p*-aminobenzoic acid is a true growth factor for *Lactobacillus arabinosus* 17-5. Purification of all the compounds exhibiting activity will be necessary to prove or disprove the specificity of *p*-aminobenzoic acid.

Addition of 1  $\gamma$  of *p*-aminobenzoic acid to 10 ml. of media based on

<sup>1</sup> Snell, E. E., and Wright, L. D., *J. Biol. Chem.*, 139, 675 (1941).

casein hydrolysates treated with charcoal in this and in other laboratories resulted in elevation of maximum acid production to, or very near to, the levels obtained with untreated hydrolysates. The results are shown in the table. In the light of these findings *p*-aminobenzoic acid should be

*Effect of Addition of 1  $\gamma$  of p-Aminobenzoic Acid per 10 Ml. to Media Based on Charcoal-Treated Casein Hydrolysates*

Hydrolysate	Charcoal used		Maximum amount of 0.1 N acid produced		
			Untreated hydrolysate	Treated hydrolysate	Treated hydrolysate + <i>p</i> -aminobenzoic acid
	Brand	Amount <i>mg. per ml.</i>	<i>ml.</i>	<i>ml.</i>	<i>ml.</i>
N.I.H.	Darco G-60	10	10.5	2.6	10.9
A	Norit A	4	*	7.7	10.4
B	Nuchar	10	*	3.6	10.5
C	Norit A	20	10.55	6.8	9.6
D	Darco G-60	10	9.8	6.6	9.5

\* Untreated hydrolysate was not available for testing.

added to the medium whenever charcoal-treated hydrolysates are used in the Snell and Wright assays for nicotinic acid or biotin.

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## CONVERSION OF ESTRONE TO ESTRIOL IN VIVO\*

Sirs:

In an endocrine-cancer study recently conducted in this laboratory,<sup>1</sup> evidence was obtained for the occurrence of estrone, estradiol, and estriol in the urines of both men and non-pregnant women. Estrone had been previously isolated from the urine of males by Dingemans *et al.*,<sup>2</sup> but the information obtained by us and others<sup>2</sup> regarding the nature of non-ketonic estrogens normally excreted by men is circumstantial. It was observed that the administration of estrone to men caused a marked increase in the excretion of all of the estrogens under discussion; isolation of the active metabolites was considered highly desirable. Accordingly, a total of 1.05 gm. of estrone acetate, m.p.<sup>3</sup> 124-125° (prepared from estrone which had been obtained from pregnant mare urine), was dissolved in oil and injected intramuscularly into healthy young men. The pooled urines (30.7 liters) which were subsequently collected were extracted with ether after acid hydrolysis. The phenols were taken up in benzene and extracted with 0.3 M sodium carbonate in order to obtain the estriol fraction<sup>4</sup> and the procedure was then repeated. Further purification of this fraction (approximately 30,000 r. u.) required partitioning between benzene and 70 per cent ethanol and treatment with phthalic anhydride. Finally, 10.3 mg. of semicrystalline material were obtained which yielded from ethyl acetate 1.6 mg. of crystals, m.p. 266-268°. Recrystallization from the same solvent gave 0.6 mg., m.p. 269-270°; a mixed melting point determination with authentic estriol,<sup>5</sup> m.p. 271-273°, gave 268-272°. The estrogenic potencies of both preparations were identical; both gave an identical spectrum in the David reaction. An additional 2 mg., m.p. 268-270°, were obtained from the mother liquors. The acetate after a

\* This investigation was aided by grants from the Committee for Research in Problems of Sex of the National Research Council and G. D. Searle and Company.

<sup>1</sup> Pineus, G., and Pearlman, W. H., *Cancer Research*, **1**, 970 (1941)

<sup>2</sup> Dingemans, E., Laqueur, E., and Muhlbock, E., *Nature*, **141**, 927 (1938)

<sup>3</sup> All melting points are corrected

<sup>4</sup> Mather, A., *Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, **133**, p. 1411 (1940)

<sup>5</sup> Pure estriol melts at 273° uncorrected or 282-283° corrected (MacCorquodale, D. W., Thayer, S. A., and Doisy, E. A., *J. Biol. Chem.*, **99**, 327 (1932-33)). Contamination with estrone may possibly account for the low melting points reported in the literature for this steroid (Doisy, E. A., and Thayer, S. A., *J. Biol. Chem.*, **91**, 641 (1931)). However, when our specimen of authentic estriol was extracted twice from benzene with equal volumes of 0.3 M sodium carbonate, 98 per cent of the estrogenic activity was removed, whereas with estrone very little or no activity was extracted (cf. Mather, foot-note 4)



single crystallization from aqueous methanol melted at 115–118°; it gave no melting point depression with authentic estriol triacetate, m.p. 124.5–125.5°, but caused a marked depression in melting point on admixture with estrone acetate, m.p. 124–125°. It is very unlikely that the estriol thus obtained arose from endogenous estrogen and not from the estrone administered, in view of the extremely low estriol<sup>1</sup> and total estrogen<sup>1,2</sup> content of urine of males.

A non-ketonic fraction was obtained after two successive treatments of the "weak" phenols with Girard's Reagent T. This material was partitioned between benzene and 70 per cent ethanol, treated with succinic anhydride, and then hydrolyzed. The brown oil thus obtained weighed 7.2 mg. Bioassay indicated the presence of an equivalent of approximately 2 mg. of  $\alpha$ -estradiol; if  $\beta$ -estradiol were the sole constituent, this fraction would have weighed about 160 mg. Further purification of this and other fractions is in progress. A more detailed report will follow.

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# THE EFFECT OF DIETHYLSTILBESTROL ON THE PLASMA PHOSPHOLIPIDS OF THE COCK (*GALLUS DOMESTICUS*)

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(Received for publication, May 9, 1942)

Lawrence and Riddle (1) in 1916 demonstrated that the blood lipids of the fowl are increased during the egg-laying season. By the administration of estrogens (2-4), either natural or synthetic, large elevations of the concentration of fats in the blood have been produced experimentally in female and male birds, or in capons. Entenman, Lorenz, and Chaikoff (4) reported a 2-fold to 4-fold increase in phospholipids alone within 48 hours after administration of diethylstilbestrol. Landauer, Pfeiffer, Gardner, and Man (5) produced concentrations of lipid phosphorus as high as 201 mg. per 100 cc. of serum after the administration of estradiol benzoate for 3 weeks. We have made further studies on the effect of the administration of diethylstilbestrol<sup>1</sup> with reference to the rate of increase of phospholipids during the course of administration and the rate of decrease when the estrogen is stopped, and have analyzed these phospholipids to determine whether and by how much the choline-containing phospholipids and the cephalins both are increased by the estrogen.

## Methods

Mature cocks of a Leghorn strain crossed with Minorea, weighing between 1.6 and 2.6 kilos, maintained in cages on a standard ration, were given 1 mg. of diethylstilbestrol dissolved in peanut oil per kilo of body weight subcutaneously, daily, for a 5 day period. They were again injected with the estrogen 33 days following the last of these injections for a 6 or 7 day period. The first group of eight birds was studied from October 29 to December 30 and the second group of seven birds from January 21 to March 18.

Specimens of blood were removed by venipuncture from all the cocks at the beginning and end of each series of injections and from smaller alternating groups as often as was practical throughout the injection and early recovery periods. The blood was heparinized and centrifuged and the plasma was analyzed for phospholipids. In many cases determinations were also made of cholesterol and neutral fat.

The plasma was extracted with 20 volumes of a 3:1 alcohol-ether mixture

<sup>1</sup> Kindly supplied by the Abbott Laboratories.

at room temperature overnight. An aliquot of this extract was ashed directly for total phosphorus content, since Man (6) has shown that greater accuracy is not attained by the more hazardous procedure involving reextraction with petroleum ether. The ashing was done with sulfuric acid and superoxol and the final phosphorus determination by the method of Fiske and Subbarow (7) adapted to the photometer. The phosphorus content was considered as one-twenty-fifth of the total amount of phospholipids.

To determine the amount of choline-containing phospholipids, an aliquot of the alcohol-ether extract was evaporated and suspended in 5 ml. of absolute methyl alcohol. Hydrogen chloride gas was bubbled through this for a period of 20 minutes to split off the choline. The alcoholic solution was evaporated to dryness at room temperature, the residue was dissolved in 6 ml. of water and centrifuged, and aliquots were taken for choline determination. The choline was then determined as the enneaiodide by the method of Roman (8) as used by Kirk (9). The procedure for splitting off the choline from the phospholipids is similar to

TABLE I  
*Comparison of Two Methods of Hydrolysis of Phospholipids*

Method	Choline containing phospholipids, mg. per 100 ml. plasma						
	Blood 1	Blood 2	Blood 3	Blood 4	Blood 5	Blood 6	Blood 7
Authors' .. ..	159	176	510	637	967	1578	2335
Thannhauser <i>et al.</i> (11).	151	172	513	592	920	1599	2260

that used by Levene (10) for hydrolysis of nucleic acid and gave results comparable to those obtained by refluxing for 3 hours with a solution of gaseous hydrogen chloride in absolute methyl alcohol as recommended by Thannhauser, Benotti, and Reinstein (11) (Table I). The content of choline-containing phospholipid was obtained by multiplying the choline value by 6.68.

The values for cephalins were obtained by difference from the amount of choline-containing phospholipids and the amount of total phospholipids. Attempts were made to separate sphingomyelin, by the methods of Kirk and of Thannhauser, Benotti, and Reinstein, for subsequent determination by phosphorus content. Neutral fat was determined by the method of Voris, Ellis, and Maynard (12) on an acetone extract of plasma, and cholesterol by the method of Liebermann and Burchard, as described by Bloor, Pelkan, and Allen (13), adapted to the photometer.

### Results

When diethylstilbestrol was administered to fifteen mature cocks daily for 5 days during the season from late October to March, the plasma

phospholipids increased continuously from a mean of 312 mg. per 100 ml. to a maximal mean value of 1952 mg. per 100 ml. 24 hours after the last injection (Table II). When administration of the estrogen was discontinued, a precipitous decrease occurred to normal levels (Fig. 1). The administration of estrogen was repeated 33 days later, this time for 6 or 7 days. The same changes occurred as before but the increases in total

TABLE II

*Plasma Lipids of Cocks Receiving Injections of Diethylstilbestrol*

Studies on Cocks 1 to 8 were made from October 22 to December 30, 1940; on Cocks 9 to 15 from January 21 to March 18, 1941. In Series 1 the cocks were injected daily for 5 days; in Series 2 Cocks 1 to 8 received seven daily injections, Cocks 9 to 15, six daily injections.

The values are expressed in mg. per 100 ml. of plasma.

Cock No.	Series 1						Series 2					
	Total phospholipids		Choline phospholipids		Cephalins		Total phospholipids		Choline phospholipids		Cephalins	
	Before	24 hrs. after	Before	24 hrs. after	Before	24 hrs. after	Before	24 hrs. after	Before	24 hrs. after	Before	24 hrs. after
1	302	3480	176	2486	126	994	264	3760	208	2285	56	1475
2	288	681	171	495	117	186	226	1190	189	612	37	578
3	337	479	188	253	149	226	281	1830	235	1120	46	710
4	236	3070	109	2144	127	926	246	3050	197	1970	49	1080
5	340	1410	183	1016	157	394	389	2105	296	1340	93	765
6	337	2190	219	1670	118	520	347	3080	253	1870	94	1210
7	253	3070	166	2309	87	761	243	3140	239	2105	4	1035
8	208	874	104	669	104	205	267	3420	225	2280	42	1140
9	417	2810	190	1775	227	1035	434	2950	301	1395	133	1555
10	285	1845	156	1080	129	765	400	1556	283	915	117	641
11	325	1390	135	777	190	613	288	1455	209	744	79	711
12	316	1860	173		143		284	2280	240	1380	44	900
13	438	2130	215	1280	223	850	250	3280	178	2150	72	1130
14	302	1063	135	695	167	368						
15	302	2930	133	1768	169	1162	338	4180	261	2675	77	1505
Mean..	312	1952	164	1316	149	643	304	2663	237	1632	67	1031
S.D. ..	60	963	35	714	41	331	66	924	39	645	34	329

phospholipid were of even greater magnitude, reaching a mean value of 2663 mg. per 100 ml. of plasma again 24 hours after the last injection. The determinations for intermediate days that were made, though on smaller numbers of the same cocks, were entirely consistent with the determinations made on the days when all the cocks were used. Although this estrogen produced an increase progressive with time in the concentration of plasma phospholipids in all the cocks, the amount of the increase in the individual cocks was highly variable. Maximal values found within

7 days, however, approached those found in 3 weeks by Landauer, Pfeiffer, Gardner, and Man when estradiol benzoate was administered to cocks, and those found in 6 days by Laskowski (14) when gonadotropic hormone of the pituitary was administered to hens. Laskowski also found the response of different individuals to be highly variable.

Both the cephalins and the choline-containing phospholipids were involved in the rapid increase in total phospholipids produced by diethylstilbestrol (Table II) and in the rapid decrease subsequent to discontinuing administration of the estrogen (Fig. 1). The mean value for choline-containing phospholipids increased from 164 to 1316 mg. per 100 ml. during the injections in Series 1 and from 237 to 1632 mg. in Series 2. The

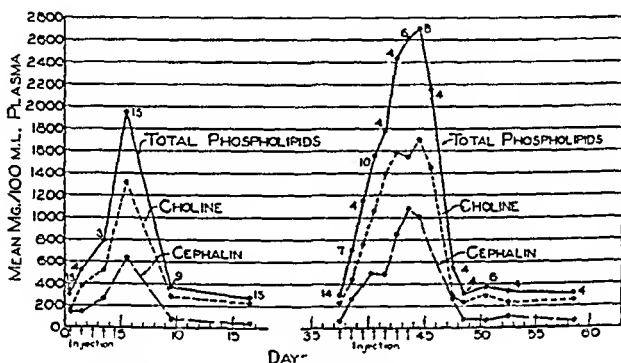


FIG. 1. Cephalins and choline-containing phospholipids in the plasma of cocks receiving 1 mg. of stilbestrol for each kilo of body weight. The numbers on the curves indicate the number of birds studied on each day. The individual values before injection and 24 hours after the last injection are given in Table II. The arrows indicate injection.

mean value for the cephalins increased from 149 to 643 and from 67 to 1031 mg. per 100 ml. of plasma during the two series of injections. An increase in both kinds of phospholipids was found in all of the birds but the extent of the increase in individual birds varied considerably. The composition of the phospholipid mixture was not altered greatly by the injections. Thus the choline-containing phospholipids averaged 52.6 per cent of the total at the beginning of the experiments, 67.4 per cent after the injections of Series 1, and 61.3 per cent for Series 2. During the recovery periods when the content of total phospholipid had returned to normal, low values for cephalins were generally found. Even 33 days after the injections in Series 1 the choline phospholipids made up 78.0 per cent of this total.

Attempts to determine what part of the choline-containing compounds

was made up of sphingomyelin were unsatisfactory, particularly when the total quantity was very high. At normal levels of total phospholipid, values for sphingomyelin which were approximately 10 per cent of the total could be obtained by either the method of Kirk or that of Thannhauser, Benotti, and Reinstein (11). However, when the total phospholipid was, for example, 2810 and the choline-containing phospholipids were 1775 mg. per 100 ml. of plasma, if the reineckate was washed only with ice-cold methyl alcohol and ether as used by Thannhauser and Setz (15) in their 1936 method and by Erickson and others (16), values of 1020 mg. for sphingomyelin were obtained. When the reineckate was washed with ice-cold acetone as recommended by Thannhauser and others (11) in 1939, a very large decrease in phosphorus content of the precipitate occurred and the extent of the decrease seemed to be dependent on the number of wash-

TABLE III

*Comparison of Concentrations of Cholesterol, Phospholipids, and Neutral Fat before and after Administration of Diethylstilbestrol*

The values are expressed in mg. per 100 ml. of plasma.

		Cock No.						
		9	10	11	12	13	14	15
Cholesterol	Before	243	223	264	230	257	227	173
	After*	900	840	595	675	694	394	925
Phospholipids	Before	417	285	325	316	438	302	302
	After*	2810	1845	1390	1860	2130	1063	2930
Neutral fat	Before	528	380	365	317	650	180	316
	After*	8320	4520	3200	4510	5230	2700	7550

\* 24 hours after five daily injections of diethylstilbestrol.

ings and on the time of washing with the ice-cold acetone, with no definite end-point. Because of such difficulties as well as the lack of information available on the nature of sphingomyelins in birds, we have been unable to determine to what extent the sphingomyelin increases, although this would be indeed important to know.

Increases in total phospholipids found 24 hours after five daily injections of the estrogen were greater than increases found in cholesterol but less than those found in neutral fat (Table III). This relation is the same as that found by Entenman, Loreuz, and Chaikoff 24 and 48 hours after administration of diethylstilbestrol.

#### *Comment*

The rapidity with which an extremely high concentration of phospholipid in the plasma of the cock may be produced by diethylstilbestrol is very

striking. In fact the action of estrogens in birds appears to be the most effective stimulus known for the production of large and rapid increases in the concentration of circulating phospholipids. It is therefore of considerable interest that the increases in phospholipids due to such a powerful stimulus as diethylstilbestrol were so variable. This may in part be due to variation in the rate of absorption, destruction, and elimination of the estrogen (17), but it may also be the result of other factors which control the concentration of circulating phospholipids. The even greater increase in neutral fat in the plasma is undoubtedly due to mobilization from the fat depots, but it would be difficult to attribute the increase in phospholipids to mobilization. Should such a process occur, the most likely tissue to be involved would be the liver. When the concentration of phospholipid increases in the plasma 10-fold in 5 to 7 days, it is almost equal to the concentration in the liver. Since the plasma volume of the bird is about 1 per cent and the liver weight 2 to 4 per cent, the total quantity of circulating phospholipid in the plasma then equals or exceeds the normal content of the liver. Although our studies of the actual content of phospholipid in the liver following administration of diethylstilbestrol are very incomplete, they do show that there is insufficient decrease of phospholipids in the liver to account for those in the blood on the basis of mobilization. It appears that the large accumulation in the blood must be due to actual synthesis, the site for which is probably the liver, as suggested by experiments of Hevesy and Hahn (18) in which radiophosphorus was administered to the laying hen.

It is interesting that when rapid production of plasma phospholipids is initiated in the cock, almost a normal partition of choline-containing phospholipids and cephalins is maintained, although the increase of the cephalins is less and more variable than that of choline-containing phospholipids. When the administration of the estrogen is discontinued, both the excess amount of choline-containing phospholipids and that of the cephalins in the circulating blood are rapidly removed. Since the phospholipids can neither be excreted nor stored in quantity as such, this suggests that both can be rapidly metabolized. After a period of administration of estrogen, the concentrations of cephalins actually decreased below the control levels and remained low for the month which elapsed before the second period of administration, after which the concentrations were again very low. The physiologic significance of this is not clear but it is perhaps in keeping with the greater variability of the cephalin fraction.

Artom and Freeman (19) found that the relatively small increases in total phospholipids of the plasma of rabbits after a fatty meal were due to increases in lecithin and not cephalin, whereas Artom (20) found that in man the increases in total phospholipids were due to increases in both lecithin and cephalin.

## SUMMARY

Concentrations of phospholipids in the plasma approaching those in the liver may be produced in cocks in 1 week by the administration of diethylstilbestrol, apparently by actual synthesis.

The concentrations of these circulating phospholipids, however, return to normal values within a few days after discontinuance of administration of this estrogen.

Both the cephalin and choline phospholipids participated in the increase of the total phospholipids produced by the estrogen; so that the ratio of choline phospholipids to cephalins was not greatly altered during the period of intense production, although a more rapid decrease of the cephalin fraction, producing a shift toward a higher percentage of choline phospholipids, was noted during the recovery period.

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# NOTE ON ELECTROPHORETIC PATTERNS FOLLOWING AERATION OF RAGWEED POLLEN EXTRACT

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While studying the effect of bubbling air through a highly pigmented solution of giant ragweed pollen extract, we found it necessary to make a careful comparison of the electrophoretic patterns obtained before and after oxygenation. Improvement in the patterns obtained for colored solutions

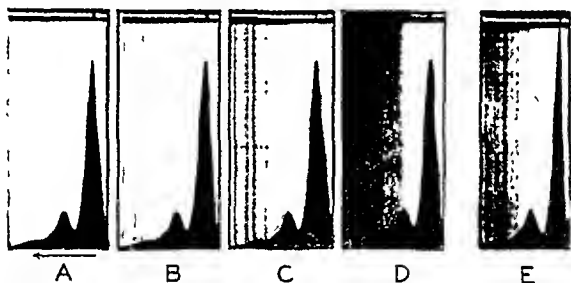


FIG. 1. Electrophoretic patterns (descending limb) of ragweed pollen extract. A through D are prints from the same negative with varying times of exposure (after aeration); E, single print (before aeration).

had previously been observed by Treffers and Moore (1) upon varying the wave-length into the infra-red region, and by Oncley (2) using filtered visible light.

Considerable facilitation in the analysis of electrophoretic diagrams obtained by the Longworth (3) technique may also be obtained by varying the exposure time of both the negative and the print. Such a procedure is an aid in identifying the pigmented or light-absorbing components and in locating deeply pigmented components which may otherwise be lost if their concentration is too low to give a detectable refractive index gradient. Fig. 1, A is an electrophoretic pattern of giant ragweed pollen extract after aeration. Compare this with Fig. 1, E which was obtained before bubbling. In Fig. 1, E the position of the main light-absorbing pigment is indicated, whereas in Fig. 1, A no absorbing pigment is observed. Fig. 1, B was ex-

posed for a longer period than Fig. 1, *A*. Fig. 1, *C*, made from the same negative as Figs. 1, *A* and 1, *B*, corresponds very closely to Fig. 1, *E* and clearly indicates the main pigmented component migrating away from the non-moving colorless component. Fig. 1, *D* illustrates a print of still greater exposure and indicates no absorption by the non-moving colorless component (4).

It is evident from our data that the analysis of electrophoretic patterns by visible light with the ordinary technique can be more successfully accomplished if various exposure times are employed in printing. In this way the different types of pigments and their photographic intensity may readily be estimated.

Ineidentally, on the basis of the data presented here it is evident that bubbling oxygen through giant ragweed pollen solution for 1½ hours does not change appreciably the electrophoretic pattern.

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# THE ARSENIC ANALOGUE OF CHOLINE AS A COMPONENT OF LECITHIN IN RATS FED ARSENOCHOLINE CHLORIDE\*

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(Received for publication, March 3, 1942)

In 1936 it was reported that the arsenic analogue of choline chloride has a marked lipotropic action in rats on a high fat-low choline diet (1), a finding confirmed by Best and Ridout (2). Later it was shown in mice that the lipotropic activity of arsenocholine chloride is at least one-half as great as that of choline chloride (3, 4). That the arsenic analogue is utilized in the biosynthesis of phospholipid was indicated by the spectrographic demonstration of the presence of arsenic in purified lecithin and sphingosine-phosphorylcholine isolated from various rat tissues (1, 4). This finding suggested that, as might be anticipated, exogenous choline may be utilized directly in the formation of phospholipid.

The lipotropic inactivity of the arsenic analogue of betaine hydrochloride was interpreted as indicating that ordinary betaine is first reduced to choline before exerting its lipotropic action (4). The recent work of Stetten (5) has shown that such a conversion occurs, although in an indirect manner: betaine yields glycine and methyl groups; the former is reduced to ethanolamine, while the latter are available for methylations, e.g. ethanolamine to choline. The lipotropic activity of betaine is now explained by its ability to serve as a source of methyl groups for the formation of choline. The failure of arsenobetaine to exert a lipotropic action indicates that this compound, unlike betaine, is unable to supply methyl groups for choline synthesis. Arsenocholine, like arsenobetaine, cannot serve as a methyl donor, since rats on a choline-free diet, containing homocystine in place of methionine, fail to grow when arsenocholine is added to the diet (6-8). Like choline, however, arsenocholine is an active lipotropic agent; thus, since its methyl groups do not appear to be labile, the lipotropic activity of this compound must depend upon reactions which involve the intact molecule of arsenocholine. Although the

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methyl groups of choline are labile, it is reasonable to conclude that its lipotropic action is related to reactions which involve the intact molecule rather than its labile methyl groups.

The spectrographic demonstration of arsenic in lecithin indicated one pathway through which the utilization of arsenocholine might conceivably lead to a lipotropic effect. However, a question as to the nature of the arsenic in the lecithin was raised by the finding (9), which we have confirmed, that rat liver forms a volatile arsenical substance, probably trimethylarsine, from the arsenic analogue of choline. The possibility was offered that the arsenic content of the lecithin was due, wholly or in part, to a degradation product of arsenocholine, rather than to a partial replacement of choline with its arsenic analogue.

It can now be stated, as a result of further experiments, not only that the feeding of arsenocholine chloride results in the appearance of significant amounts of arsenic in the choline fraction of a lecithin hydrolysate, but also that this arsenic is almost certainly in the form of arsenocholine.

#### EXPERIMENTAL

A group of rats was fed a diet containing arsenocholine chloride (1 per cent) for a period of 1 week. It should be noted that these animals showed no signs of toxic reactions. The combined carcasses, exclusive of the alimentary tracts, were weighed (1460 gm.), ground, and extracted repeatedly with hot ethanol (95 per cent). Phospholipids were separated from the ethanol by acetone precipitation (yield, 8.9 gm.) and hydrolyzed with 10 per cent hydrochloric acid (100 cc.) at 100° for 18 hours. Fatty acids were removed by filtration (0°) and the filtrate diluted to 500 cc. A filtered solution of ammonium reineckate (saturated at 50°) was added in excess (250 cc.) and the precipitate recovered by filtration. Repeated extraction of the precipitate with absolute ethanol, until a colorless extract was obtained, served to remove certain impurities (10); aminoethanol does not form a precipitate with ammonium reineckate (11). The residue was dried *in vacuo* over phosphoric anhydride to constant weight (1.77 gm.). The various fractions were subjected to analysis for arsenic, according to the procedure of Morris and Calvery (12); see Table I.

It is clear that essentially all the arsenical compounds present were retained in the choline fraction (the "purified" reineckate precipitate).

A sample of the reineckate precipitate (1.125 gm.) was decomposed according to the method of Kapfhammer and Bischoff, as described by Mann and Quastel (13), and the solution obtained was reduced in volume to 10 cc. Of this solution, 9 cc. were treated with a 25 per cent solution of gold and sodium chloride (5 cc.). The resultant crystalline precipitate was recrystallized from dilute hydrochloric acid (2 per cent) containing

gold and sodium chloride (0.7 per cent). A sample (0.185 gm.) of the yellow, needle-like crystals obtained was analyzed for nitrogen, arsenic, and gold; see Table II. On the basis of the amounts of arsenocholine and choline aurichlorides required by the arsenic and nitrogen content, the gold content should have amounted to 79.9 mg.; 80.8 mg. were found (+1.2 per cent).

Additional evidence that the arsenic content of the crystalline aurichloride is actually due to arsenocholine is offered by the following highly specific, but qualitative, test. A sample of the aurichloride (9.25 mg.)

TABLE I  
*Arsenic Content of Phospholipid Fractions*

Fraction	Arsenic	
	mg.	per cent of total
Aqueous filtrate from reineckate ppt..	0.55	1.73
Alcoholic extract of " "	0.89	2.80
"Purified" reineckate ppt. (1.77 gm.)	30.30	95.46

TABLE II  
*Arsenic, Nitrogen, and Gold Content of Crystalline Aurichloride (185 Mg.)*

Element	Found	Equivalent weight of gold	Equivalent compound	Theoretical weight of equivalent compound
	mg.	mg		mg.
N	5.41	76.16	$C_5H_{14}ON \cdot AuCl_4$	171.16
As	1.41	3.71	$C_5H_{14}OAs \cdot AuCl_4$	9.49
Au	80.8			
Theoretical weights from N and As.		79.87		180.65

was dissolved in water and treated with powdered silver to precipitate the gold (14). The combined filtrate and washings (20 cc.) were made alkaline with sodium bicarbonate; iodine-potassium iodide solution (13) was then added (29 cc.). The precipitate, incompletely recovered by centrifugation, was dissolved in chloroform and the solution extracted with dilute hydrochloric acid (2 per cent) to recover any water-soluble material. The amount of arsenic found in the extract was small (0.017 mg.); it was equivalent, however, to about one-fourth of the theoretical amount. Although techniques were employed which permitted losses to occur, a fraction was obtained under conditions highly specific for choline. The occurrence of arsenic in this fraction, and the fact that it was derived from

a crystalline gold salt which analyzed almost theoretically for a mixture of choline and arsenocholine aurichlorides in a ratio of 18:1, give strong indication that choline and its arsenic analogue were in admixture.

The techniques used in the isolation of the various choline fractions are dependent on well established procedures. The insolubility of choline reineckate in water and in absolute ethanol has served as the basis for several methods for the determination of choline, particularly those of Beattie (15), Jacobi, Baumann, and Meek (16), and Thornton and Broome (11). We<sup>1</sup> have found that 100 cc. of distilled water at 23° dissolve 0.041 gm. of choline reineckate; under similar circumstances 0.064 gm. of arsenocholine reineckate is dissolved. Strack and Schwaneberg (17) observed that excess ammonium reineckate and cooling reduced the solubility of choline reineckate from 0.019 per cent (at 18°) to 0.0015 per cent; undoubtedly a similar effect is exerted on arsenocholine reineckate. In absolute ethanol choline reineckate is soluble to the extent of 0.018 gm. per 100 cc. at 23°, while 100 cc. of a saturated alcoholic solution of arsenocholine reineckate contains 0.038 gm. The data shown in Table I demonstrate that over 95 per cent of the arsenic present in the various fractions of the lecithin hydrolysate was contained in that portion of the reineckate precipitate which was insoluble in absolute ethanol. Also, on the basis of the work of Jacobi *et al.* (16) it may be assumed that essentially all the choline (and therefore all the arsenocholine) was present in the "purified" reineckate precipitate; if the latter consisted entirely of arsenocholine and choline reineckates, the ratio of the former to the latter, expressed as the chlorides, was 1:6.4.

In the procedures leading to the formation of the *recrystallized* gold salt it is probable that fractionation occurred at the expense of arsenic, since the calculated ratio of arsenocholine to choline, expressed as the chlorides, was in this case 1:14.2. Such fractionation is doubtless related to the fact that arsenocholine aurichloride is more soluble than is choline aurichloride (a saturated aqueous solution (23°) of choline aurichloride was found to contain 1.52 gm. per 100 cc., while under similar conditions 2.45 gm. of arsenocholine aurichloride were found).

Precipitation as the insoluble periodide has been used by several workers (18, 12, 19, 20) as a means for the determination of choline. Although the iodine complex is very unstable, and reliable analytical results are difficult to obtain, the formation of the complex lends itself, under certain conditions, to the precipitation of choline with a high degree of specificity. That arsenocholine forms an iodine complex similar to that of choline is indicated by the successful use by Roepke and Welch (21) of the periodide

<sup>1</sup> The authors are grateful to Miss Ethol Shiels, of the Medical Research Division of Sharp and Dohme, Inc., for assistance with the solubility determinations.

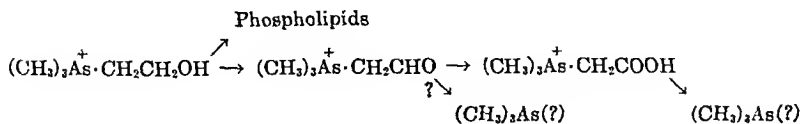
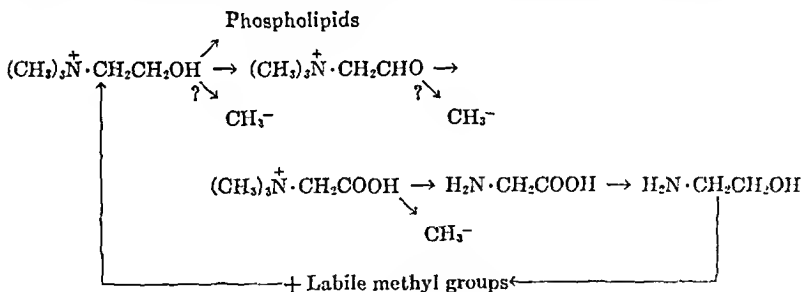
procedure for the determination of arsenocholine. The complex is altogether too unstable to permit solubility determinations to be made.

Incontrovertible proof that the arsenic component is actually the arsenic analogue of choline requires the separation of that substance from choline, and the positive identification of the pure compound; however, the difficulties attending such separation appear to be great.

## DISCUSSION

The work of Perlman and Chaikoff (22) with radioactive phosphorus has shown that choline markedly stimulates the turnover of phospholipids. Stetten's investigations with choline containing heavy nitrogen (5) have offered convincing evidence for the direct synthesis of phospholipids from ingested choline. The evidence here presented indicates that the arsenic analogue of choline may, to a certain extent at least, replace choline in the biosynthesis of lecithin. Studies with the triethyl homologue of choline, triethyl- $\beta$ -hydroxyethylammonium chloride, failed to show that this compound can be utilized for lecithin synthesis (23).

The different pathways of metabolism of choline and its arsenic analogue are presented in the accompanying series of reactions; stages concerning which the evidence is incomplete are indicated by question marks.



The biological significance of the conversion of choline to betaine aldehyde and betaine (liver, kidney) is not at present understood. It has been suggested (24) that such oxidation occurs prior to demethylation; however, betaine is less active than choline as a lipotropic agent (2, 4), as an accelerator of phospholipid turnover in the liver (25), as a donor of



methyl groups to homocysteine (6, 8), and, as an antiperotic agent in chicks, it is inactive (26). Semiquantitative studies of a nature similar to those with betaine have not yet been reported with betaine aldehyde; it has been found, however, that it does not possess antiperotic activity and stimulates growth but slightly in chicks;<sup>2</sup> preliminary results in rats<sup>3</sup> indicate that it is definitely less active than choline, both as a lipotropic and as a renal antihemorrhagic agent. Oxidation of choline does not appear to be preliminary to the exertion of a lipotropic action, since betaine aldehyde and betaine are less active than choline, while arsenobetaine (4) and probably arsenobetaine aldehyde are inactive,<sup>3</sup> despite the activity of arsenocholine.

Rat liver rapidly oxidizes choline or its arsenic analogue to the corresponding aldehyde; conversion to the corresponding betaine proceeds more slowly. The total oxygen consumed is equivalent to an uptake of 2 atoms per molecule of choline or arsenocholine. Unlike betaine aldehyde, arsenobetaine aldehyde has not been isolated; it has been shown, however, that during the oxidation of arsenocholine by rat liver a compound is formed which reacts with 2,4-dinitrophenylhydrazine; this reactive compound gradually disappears as the oxidation continues. When semicarbazide is added, the oxidations both of choline and of arsenocholine may be arrested at the aldehyde stage. In the case of the arsenic analogue a secondary reaction takes place which leads to the production of a strong garlic-like odor suggestive of trimethylarsine; in addition, acid permanganate placed in the side arm of the oxidation chamber is decolorized, indicating the formation of a volatile reducing substance (9). It should be noted that significant amounts of trimethylamine, or other volatile substances capable of reducing acid permanganate, do not appear to be formed during the oxidation of choline by rat liver. The formation of the volatile arsenical substance is blocked by the addition of semicarbazide (9), a finding which presumably indicates that cleavage of the analogue occurs following its oxidation to the aldehyde. Rupture of the molecule appears to occur *in vivo* as well as *in vitro*, since the exhalations and tissues of animals fed arsenocholine chloride or arsenobetaine hydrochloride have a marked garlic-like odor. The absence of toxic effects is clear evidence that the volatile substance is not arsine, or its mono- or dimethyl derivatives; preliminary experiments with trimethylarsine produced no evidence of toxicity in the dosage used. Attempts to recover the volatile arsenical from tissues in sufficient amount for identification were not successful; the substance was found, however, to be wet-ashed with difficulty and to give reactions with mercuric chloride paper which were not typical

<sup>2</sup> Jukes, T. H., and Welch, A. D., in preparation.

<sup>3</sup> Welch, A. D., unpublished research.

of arsine. The evidence, though inconclusive, suggests that arscnobetaine and possibly arscnobetaine aldehyde undergo cleavage to form trimethylarsine.

The N:C bonds between N and  $\text{CH}_3$  in choline, betaine aldehyde, and betaine are possibly all subject to disruption, while that between N and the 2-carbon side chain appears to be more stable. With the arsenic analogues the As:C bonds between As and  $\text{CH}_3$  appear to be little, if at all, subject to rupture, while the bond between As and the 2-carbon side chain is more readily split. Clearly, the utilization of arsenocholine in the biosynthesis of lecithin, its inactivity as a methylator of homocysteine, and its marked activity as a lipotropic (1, 2, 4) and renal antihemorrhagic (27) agent suggest that it is biologically active only as an intact molecule. Logically, therefore, choline also functions as a lipotropic and renal antihemorrhagic agent by means of reactions involving the intact molecule. Additional evidence for this concept is afforded by the lipotropic (28, 23, 27) and renal antihemorrhagic (27) actions of the triethyl homologue of choline and the similar activities (27) of the diethylmonomethyl homologue, although both were found to be inactive as methyl donors (6, 8).

With regard to the renal antihemorrhagic action of arsenocholine, and the two homologues mentioned above, an alternative view should be considered. The lipotropic action of these compounds might "spare," for the protection of the more sensitive kidney tissue, small amounts of choline formed as a result of the synthetic activity of the young rat. Conceivably, this protection might be accomplished by some mechanism other than that which operates through utilization of the synthesized choline as an intact molecule, perhaps in a manner similar to that which has been proposed by Jacobi and Baumann (24). Such a view, however, is not in agreement with the interpretation of preliminary data obtained from experiments now in progress.

The information available would suggest that fatty livers develop when there is a deficiency in the amount of choline available for synthesis of compounds concerned with lipid transport, and that hemorrhagic kidneys may result from an acute deficiency in the synthesis of compounds concerned with cell structure, at a critical period in the growth of the rat. It is not necessary to assume that the synthetic reactions involved in each case are in every detail identical. In fact, preliminary findings indicate that differences do exist between lipotropic and renal antihemorrhagic activity, and Jukes (26) and Jukes and Welch<sup>2</sup> have found that certain choline derivatives are ineffective as antipercotic agents in fowls, although they have marked lipotropic or renal antihemorrhagic activity in rats.

Although choline is of great importance as a source of methyl groups, it seems probable that one of its functions, lipotropic action, depends on reactions which involve the intact molecule. Whether a similar

conclusion may be applied eventually to the renal antihemorrhagic and antiperotic actions of choline is not at present certain.

#### SUMMARY

Evidence is offered that arsenocholine may substitute for choline in the biosynthesis of lecithin. The reineckate and gold salt fractions, obtained from rats fed arsenocholine chloride, yield analytical data which indicate that the arsenic in such lecithin is in the form of the arsenic analogue of choline.

The significance of this finding is discussed with reference to the products of metabolism of choline and of its arsenic analogue, and with reference to the view that the intact molecule of choline, rather than its labile methyl groups, is responsible for its lipotropic action (and possibly for its renal antihemorrhagic and antiperotic actions).

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# THE MOLECULAR SIZE AND SHAPE OF THE NUCLEIC ACID OF TOBACCO MOSAIC VIRUS

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The asymmetric nucleoprotein of tobacco mosaic virus has a molecular weight of about  $4 \times 10^7$  (1) and approximate molecular dimensions of  $150 \text{ \AA.} \times 2800 \text{ \AA.}$  (2). 5 to 6 per cent of the virus is a ribonucleic acid (3) which may be separated from the protein by many methods (4-8). A recent report by Schramm that the separation may also be effected by a nucleophosphatase derived from calf intestinal mucosa (9) has not been confirmed by the present authors (10). The virus does not appear to be a protein nucleate of the type described by Longworth and MacInnes (11), since the nucleic acid cannot be separated by means of electrophoresis over a wide pH range (12). The absence of a salt-like linkage has also been noted in various streptococcal nucleoproteins (13), and nucleoproteins of this type may be considered to be a special case of a large group of biological compounds; namely, enzymes, viruses, and other proteins which possess phosphorylated prosthetic groups in an unknown mode of linkage. The investigation of tobacco mosaic virus structure by means of x-ray analysis (14) and electron microscopy (2) has not indicated concentration of the comparatively dense nucleic acid in any particular part of the nucleoprotein molecule. It was considered that a study of the size and shape of the nucleic acid which could be obtained from the virus might give some information as to the nature of its distribution and its mode of union to the protein.

Thymus or desoxyribonucleic acid, in contrast to ribonucleic acid, shows an extreme dependence of the degree of polymerization on its concentration in solution. The conclusions derived from kinetic studies undertaken by numerous investigators (15-18) have been in agreement; namely, that desoxyribonucleic acid polymerizes polydispersely in solution to form heavy asymmetric particles. Estimates of the size, shape, and state of dispersion of this material at infinite dilution are unavailable at present.

The physical data on ribonucleic acid are rather meager. This material has generally been isolated from various tissues after treatment with alkali. Myrbäck and Jorpes (19) have estimated from diffusion measurements that a commercial yeast nucleic acid possessed a molecular weight

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lated from the height-area and the inflection point methods agreed in the case of the Nucleates B and C to within 4 per cent. The theoretical curves calculated from the average diffusion constants were almost exactly congruent to the experimental curves. When the values for diffusion constants calculated by the two methods did not agree to within 4 per cent, the curves were analyzed by the method of moments (28), which yielded average diffusion constants. The diffusion of the Nucleate A gave abnormal, but symmetrical, curves at concentrations up to 0.6 per cent. The polydispersity which was indicated by these latter analyses was not apparent in the

TABLE II  
*Physical Constants, Size, and Shape of Virus Nucleates A, B, and C*

Preparation No	Freshly isolated Nucleate A			Spontaneously decomposed Nucleate B			Alkali-treated Nucleate C
	1	2	3	1	2	3	
pH of analyzed solutions	4.9	4.9	4.9	6.8	6.8	4.9	6.8
Sedimentation constant, $S_{20} \times 10^{-13}$	7.7	8.6*	8.3	3.41	5.79	4.53	2.41
Diffusion constant, $D_{20} \times 10^{-7}$		2.44*	1.63	3.32	5.22	3.57	9.39
Intrinsic viscosity $[\eta]$ ...	62.4	62.4			27.8		12.8†
Asymmetry (axial ratio)							
Viscosity	27	27			16		9.7
$S_{20}$ , $D_{20}$		32*	61	40	13	31	9.7
Molecular weight $\times 10^3$							
$S_{20}$ , $[\eta]$	150	180*					15
" $D_{20}$		200	290	59	63	70	15
Osmotic pressure			$\gg 60$		59	54	16†

\* These values are derived from data extrapolated to infinite dilution.  $S_{20}$  values at 0.36, 0.60, and 1.0 per cent were 7.5, 6.6, 5.9, respectively;  $D_{20}$  values at 0.36 and 0.60 per cent were 2.34 and 2.06, respectively.

† These data were obtained in a buffer consisting of 0.1 M  $(\text{NH}_4)_2\text{SO}_4$  and 0.01 M acetate at pH 4.9, which was used for all analyses obtained at that pH. The veronal buffer at pH 6.8 described previously was used in the remaining determinations.

curves obtained in the diffusion of the spontaneous decomposition products derived from this polydisperse material. The results are summarized in Table II.

*Sedimentation*—Preparations of Nucleates A, B, and C were examined by Dr. M. A. Lauffer in an analytical ultracentrifuge (29, 30), equipped with a Svensson-Philpot optical system (31). The sedimentation constants obtained in solutions of 0.1 or 0.2 ionic strength were calculated from the position of maximal height in the approximately symmetrical boundaries. Single boundaries were always observed but the correlation of boundary spread and diffusion was not determined. Nucleate A was clearly inhomogeneous.

gencous; the degree of homogeneity in Nucleates B and C was considerably greater, and sharp boundaries were obtained which spread quite slowly. As the decomposition of Nucleate A progressed from week to week at pH 5, the sedimentation curve was displaced with a gradual decrease in  $S_{20}$ , till the asymptotic  $S_{20}$  of Nucleate B was obtained. No resolution of Nucleate A or B was obtained during this decomposition despite considerable differences in  $S_{20}$ . The sedimentation constants obtained for the different preparations are given in Table II.

*Osmotic Pressure*—Measurements were made in the osmometer devised by Northrop and Kunitz (32), at 4° in buffered solutions of 0.1 and 0.2 ionic strength. Measurements obtained in solutions of molar ammonium sulfate were indicative of larger molecular weights. The hydrostatic pressure produced by Nucleate A was inadequate for accurate measurements; in the case of Nucleates B and C, pressures of 50 to 100 mm. of water were obtained, depending on the concentration used. Effective concentration was estimated from the phosphorus content inside and outside of the collodion membrane. Calculations of molecular weight were made, assuming the validity of van't Hoff's law in the systems investigated (33). The data are given in Table II.

*Viscosity*—Viscosity measurements were made at 25.1° in a special Ostwald viscometer designed by Lauffer to keep the rate of flow low and hence to reduce partially the orienting influences which produce anomalous viscosity (33, 34). Calculations of asymmetry were made according to the Simha equation relating intrinsic viscosity and axial ratio (35). Intrinsic viscosity is defined as the specific viscosity divided by the particulate volume fraction per gm., in the range of linear proportionality between specific viscosity and concentration (33). The assumption was made that the particles behaved as rigid rods, rather than as disks.

The viscosity data which are presented in Fig. 1 and Table II show that the asymmetries of the preparations are in the order,  $A > B > C$ . Preparation 2 of Nucleate C in Fig. 1 was obtained by treatment of a mixture of Nucleates A2 and B2 with 5 per cent sodium hydroxide for 2 hours at 4°. The viscosity of this material was determined after neutralization with acetic acid, dialysis against distilled water, and adjustment to the proper concentration in veronal buffer at pH 6.8. Osmotic pressure measurements demonstrated the low molecular weight of Nucleate C2. Material obtained in this manner, therefore, corresponded exactly to the Nucleate C isolated from alkali-denatured virus.

Preparations of Nucleate A were examined approximately 1 week after heat denaturation of the virus, while preparations of Nucleate B were examined after approximately 1 month of spontaneous decomposition. Preparations of the same number in Fig. 1 were from the same batch of

virus. Nucleates A1, A2, B2, and C in Table II correspond to Preparations A1, A2, B2, and C4, respectively, in Fig. 1.

*Optical Properties*—Neutral gels of Nucleate A, when examined by means of a polarizing microscope, showed spontaneous birefringence, while gels of Nucleate C and alkaline gels of Nucleate A did not possess this property.

*Estimations of Molecular Weight and Asymmetry*—Molecular weights

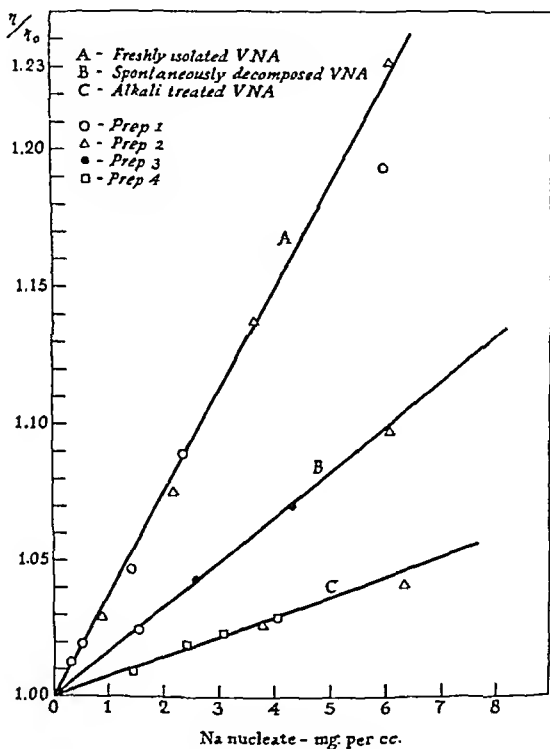


FIG. 1. Relative viscosity of solutions of virus ribonucleate (VNA)

were calculated from sedimentation, diffusion, and partial specific volume data, by means of the Svedberg equation. Calculations of asymmetry, assuming rod-shaped non-hydrated particles, were made from the frictional ratios. With the same assumptions, frictional ratios were estimated from the asymmetry determined by means of viscosity data. Molecular weights were also calculated (17) from these frictional ratios and the sedimentation or diffusion constants.

The molecular weights and asymmetries of Nucleates B, C, and Preparation 2 of Nucleate A probably represent materials dispersed at infinite dilution. The diffusion constants of Nucleate C at 0.1 and 0.7 per cent were approximately the same, permitting the use of data obtained at 0.7 per cent as data extrapolated to infinite dilution. The sedimentation constants of Nucleate B in the range of 0.4 to 1.0 per cent were approximately the same, permitting the use of data obtained at a single concentration within this range as data extrapolated to infinite dilution. In the case of Nucleate A, both sedimentation and diffusion constants varied as a function of concentration. The values of  $S_{20}$  and the frictional ratios determined at specific concentrations and extrapolated to infinite dilution were then used in the estimation of molecular weight and asymmetry for Preparation 2 of Nucleate A. The extrapolation to infinite dilution is implicit in the estimation of intrinsic viscosity. These calculations and the data on which they are based are assembled in Table II.

*Electron Microscopy*—Preparations of Nucleates A and B were examined in the electron microscope by Dr. T. F. Anderson, RCA Fellow of the National Research Council. Particles of these nucleates were invisible in the electron micrographs obtained; hence, the conclusion may be drawn that two dimensions of the particles are no greater than the present lower limit of resolution of the instrument, which is considered to be about 30 to 50 Å.

#### DISCUSSION

The molecular weights and asymmetries of the three nucleates determined by various independent methods are in agreement despite the many assumptions used and the state of development of the theory on the behavior of elongated particles in solution. The evidence that molecularly dispersed nucleic acid approximates a tetranucleotide in size is quite scanty (36, 37). Recent physical evidence on this question, as well as that presented in this paper, is in opposition to this view. The molecular weight of about 15,000, which was found for the degraded Nucleate C, indicated about 45 nucleotides per molecule. Furthermore, the postulated purine to pyrimidine ratio of 2:2 has not been thoroughly proved. Loring found that the virus nucleic acid contained less purine than was to be expected, assuming this theoretical ratio. The nitrogen to phosphorus ratio in this material has consistently been less than the theoretical value of 3.75, a fact which might be expected from Loring's purine data. In any case, there is at present no evidence that a unit smaller than 15,000 and larger than a nucleotide exists as a fundamental unit of the virus nucleic acid.

It is considered that of the three preparations of Nucleate A described in Table II, Preparation 3 most closely approximated the undecomposed Nucleate A. Extrapolation of the data on Preparation 3 to infinite dilution



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# REACTIONS OF 2-METHYL-1,4-NAPHTHOQUINONE WITH WHOLE BLOOD AND PLASMA STUDIED BY MEANS OF A RAPID COLORIMETRIC METHOD

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Recently Seligman and coworkers (1) reported that vitamin K<sub>1</sub> has such a persistent and prolonged action that a single small dose is adequate for the treatment of even severe cases of hypoprothrombinemia, and may well do the work of repeated doses of other related antihemorrhagic agents. This point has been emphasized by Fieser (2). In the course of our work on the determination of vitamin K<sub>1</sub>, it was found that the vitamin is remarkably stable in whole blood (3). More recently we reported that 2-methyl-1,4-naphthoquinone reacts with whole blood, and, unlike vitamin K<sub>1</sub>, this substance causes a marked methemoglobin formation (4). A rapid method for the determination of the methylnaphthoquinone has now been devised and used to study the reactions of this compound with whole blood and plasma.

## Method

To 10 cc. of a solution containing the naphthoquinone, 4 cc. of cysteine solution<sup>1</sup> (25 mg. per cent) and 1 cc. of N sodium hydroxide are added. Within 15 minutes, an intense, stable, yellow color is formed. These colored solutions absorb maximally at 4400 Å. as shown in Fig. 1. Although a filter transmitting maximally at this wave-length would ordinarily be desirable, a blue filter with maximum transmittance at 4050 Å. has been used to calibrate the Evelyn colorimeter against solutions containing 1 to 10  $\gamma$  of the naphthoquinone per cc.

Determinations can be performed rapidly within an error of  $\pm 3.6$  and a maximum deviation of 10 per cent. The same results are obtained whether the naphthoquinone is dissolved in water or alcohol, or mixtures of the two solvents. The addition of aqueous reagents to alcoholic blood filtrates causes turbidity due to the precipitation of lipid material. This is avoided by adding 3 cc. of ether to 10 cc. of alcoholic filtrate followed by 1 cc. of cysteine hydrochloride solution (100 mg. per cent) and 1 cc. of 0.1

<sup>1</sup> Fieser reported (2) that cysteine reacts with 2-methylnaphthoquinone to give a highly colored product.

N sodium hydroxide. The same calibration curve is obtained and the accuracy of the method remains unaltered (Fig. 2). The method is directly applicable to the determination of the water-soluble, sodium 2-methyl-1,4-naphthoquinone-3-sulfonate. When the factor 0.628 was used for conversion of the sulfonate to terms of free methylnaphthoquinone, triplicate determinations gave 100 per cent ( $\pm 1$ ) recoveries. The simplicity, reproducibility, and flexibility of the method are notable.

We have not extensively investigated the nature of substances which may interfere in this test, since none is normally found in whole blood filtrates

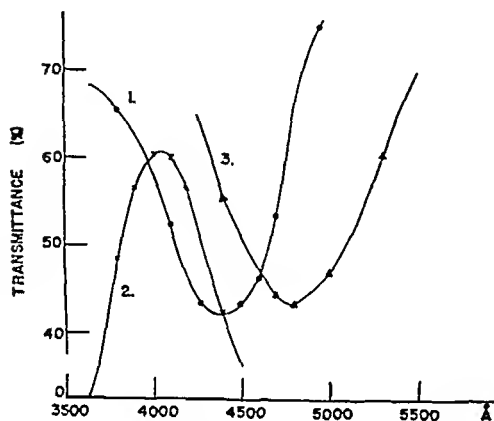


FIG. 1

FIG. 1. Relative transmittance of a solution of the cysteine color reaction (Curve 1), the No. 400 filter (Curve 2), and an alkaline solution of phthiocol (Curve 3).

FIG. 2. Calibration of the Evclyn colorimeter equipped with a No. 400 filter. The transmittance is plotted against micrograms of 2-methyl-1,4-naphthoquinone per cc. of solution. The color was developed in aqueous solutions (O), in alcoholic solutions (+), or in alcohol-ether solutions ( $\Delta$ ).

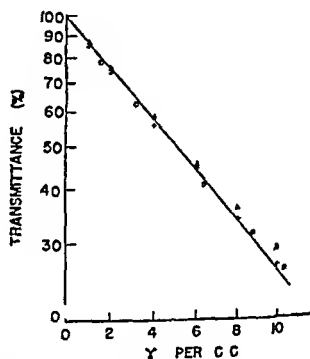


FIG. 2

or plasma. Unlike 2-methyl-1,4-naphthoquinone, the 2,3-dimethyl derivative does not give the color reaction. Large concentrations of vitamin K<sub>1</sub> give a slight amount of color. Phthiocol interferes only in that it imparts a red color to the solution when alkali is added. This red color, which absorbs sharply at 4750 Å. (Fig. 1) is more efficiently removed by a No. 400 than a No. 440 filter.

*Experiments with Plasma*—Recovery experiments were performed as follows: Varying amounts of the methylnaphthoquinone dissolved in 0.5 cc. of 50 per cent alcohol were added to 10 cc. portions of a fresh sample of plasma. At the time intervals stated in Table I, aliquots were withdrawn

and analyzed directly without deproteinization as follows: 1 cc. of plasma was diluted to 10 cc. with water, and 4 cc. of cysteine solution (25 mg. per cent) and 1 cc. of N sodium hydroxide were added. The control sample, containing no naphthoquinone, gave no color and was used to indicate 100 per cent transmittance.<sup>2</sup> The remaining samples were read then in the Evelyn colorimeter.

The recovery of added methylnaphthoquinone falls within 5 per cent of the theoretical if the analyses are performed within 30 minutes after the addition of the methylnaphthoquinone to the plasma (Table I), but the recovery diminishes with time. When the test was performed at the 5 hour interval, a pronounced red tint was observed in the test solutions.

This red color, uncomplicated by the cysteine reaction, was studied directly by omitting the cysteine from the test procedure. Control solutions of the methylnaphthoquinone in water remained essentially colorless, whereas the plasma samples developed a red color. The intensity of this

TABLE I

*Recovery of Added 2-Methyl-1,4-naphthoquinone from Blood Plasma*

The values are expressed in micrograms per cc. of plasma.

Sample No.	Added 2-methyl-1,4-naphthoquinone	Recovery		
		0.5 hr.	1 hr.	5 hrs.
1	0	0	0	0
2	53	53	37	24
3	95	99	78	70
4	177	173	158	151

color increased with the naphthoquinone concentration. The color development reached a maximum 15 minutes after the addition of the alkali and the depth of color was the same whether the plasma was treated with alkali 15 or 60 minutes after the preparation of the plasma samples. The compound responsible for this red color shows the properties of phthiocol in that it is reversibly reduced by sodium hydrosulfite; it is red in alkaline solution from which it can be removed by dialysis; it is yellow in acid solution from which it can be extracted with ether; and it is extracted from ether solutions by sodium carbonate solutions to which it imparts a characteristic red color.

Since the isolation of the minute amount of phthiocol from the plasma

<sup>2</sup> The same general procedure has been used throughout. A single sample of blood was used for each series of experiments. An aliquot used for control purposes was treated as in the test samples with the single exception that no methylnaphthoquinone was added. These control samples were always used to indicate 100 per cent transmittance.

samples is impracticable, model experiments were performed.\* The identity of the product was established as follows: To a liter of water saturated at room temperatures with the methylnaphthoquinone (120 mg.), 20 gm. of glucose and sodium hydroxide to a final concentration of 0.15 N were added. After 45 minutes of occasional shaking, the solution was acidified, extracted with ether, and the phthiocol was removed from the ether phase with dilute sodium carbonate solution. The carbonate solution was acidified and the phthiocol was again removed with ether. The ether was washed and dried, and, on evaporation of the ether, about 40 mg. of product were obtained. Recrystallized from acidulated water and then from hexane, the product melted at 171–172° and the melting point was not depressed when the sample was mixed with a pure specimen of phthiocol.

$C_{11}H_8O_2$ . Calculated, C 70.26, H 4.29; found, C 70.56, H 4.29

The effect of alkali and glucose, a normal constituent of plasma, upon the rate of conversion of the methylnaphthoquinone to phthiocol was studied. By means of the same extraction procedures employed in the isolation experiment, the rates were followed by the development of the red color with time. It was found that, in solutions of 0.01 and 0.1 N sodium hydroxide at room temperature with constant shaking, the methylnaphthoquinone is oxidized to phthiocol, and that the rate of oxidation is markedly accelerated by the presence of 1 per cent glucose.

From these model experiments it would appear that the product formed on the addition of alkali to samples of plasma containing the methylnaphthoquinone is phthiocol. It would seem reasonable to suppose that the product formed in the plasma samples at physiological pH values, though generated more slowly, would be the same. However, the over-all reaction with plasma is not as simple as this. A major portion of the naphthoquinone interacts with the plasma proteins as shown in the following experiments.

Varying amounts of the naphthoquinone (0, 0.5, 1.0, and 2.0 mg.) dissolved in 0.5 cc. of 50 per cent alcohol were added to 10 cc. portions of a freshly drawn sample of plasma, and these were stored in the dark at room temperature. Aliquots were withdrawn at the time intervals stated in Table II, and analyzed as follows: 1 cc. of plasma was pipetted into 9 cc. of absolute alcohol and after 5 minutes the proteins were separated by centrifugation. The proteins were washed with 2 cc. of alcohol and centrifuged, and again with 3 cc. of absolute ether. Supernatant liquids were decanted through the same dry filter. The filtrate volume was adjusted to 13 cc. with ether and 1 cc. of cysteine solution (100 mg. per cent) and 1 cc. of 0.1 N sodium hydroxide were added. The colors were read as usual.

The results (Table II) indicate that the naphthoquinone is rapidly ad-

sorbed by, or undergoes addition reactions with, the plasma proteins. The protein precipitates from the 5 and 15 minute samples were redissolved in water and the precipitation was repeated, but none of the missing naphthoquinone was recovered. This would seem to indicate that the methyl-naphthoquinone is not bound to the proteins in a simple reversible type of adsorption. Although it may be supposed that the alcoholic precipitation may denature the proteins and thus produce irreversible changes, 2,3-dimethyl-1,4-naphthoquinone and vitamin K<sub>1</sub> are quantitatively recovered from alcoholic filtrates of whole blood or plasma. The linkage of the naphthoquinone to the plasma proteins is reversed by the cysteine reaction. When the protein precipitates from the 5 and 15 minute samples were redissolved in water, and tested directly by the cysteine reaction, practically all of the missing naphthoquinone was recovered (see also Table I). However, sodium 2-methyl-1,4-naphthoquinone-3-sulfonate<sup>3</sup> gives the cysteine

TABLE II

*Recovery of Added Methyl-naphthoquinone from Blood Plasma following Protein Precipitation*

The values are expressed in micrograms per cc. of plasma.

Sample No.	Added methyl-naphthoquinone	Recovery				
		5 min.	15 min.	1 hr.	4 5 hrs.	22 hrs.
1	0	0	0	0	0	0
2	47.6	32	28	18	10	5
3	95.2	65	59	43	38	28
4	190.0	130	124	116	111	85

reaction as the result of the replacement of 3-sodium sulfonate grouping. A union of the methyl-naphthoquinone with the plasma proteins seems to be implicated.

As previously noted (4) a slow, irreversible change occurs in the course of the reaction of the methyl-naphthoquinone with plasma, and this change involves an air oxidation which produces a yellow color. Addition of alkali changes this color from yellow to red. The intensity of color imparted to the plasma is proportional to the quinone concentration and increases with time. The product which yields this color does not give the cysteine reaction. It is reversibly reduced by sodium hydrosulfite. It is not separated in acid, neutral, or alkaline solution from the plasma proteins by dialysis. It remains with the protein upon alcoholic precipitation and

<sup>3</sup> The structure assigned by Moore (5) to this material (hykinone) has recently been criticized by Baker *et al.* (6). These workers report that the strongly antihemorrhagic bisulfite addition compound of 2-methyl-1,4-naphthoquinone does not possess a sodium sulfonate grouping in the 3 position.

goes back into solution when the proteins are redissolved in water. Thus, the water-insoluble naphthoquinone has been converted to a water-soluble substance, and at this stage the naphthoquinone, clearly, is linked to the proteins.

*Experiments with Whole Blood*—Aqueous solutions of sodium 2-methyl-1,4-naphthoquinone-3-sulfonate<sup>3</sup> were added to oxalated samples of whole blood, and aliquots were centrifuged 15 and 60 minutes after the preparation of the solutions. 1 cc. samples of the plasma, analyzed directly without protein precipitation, gave recoveries of 116 ( $\pm 3$ ) per cent, indicating that this product is not distributed equally between the plasma and cells. Methemoglobin is slowly formed, becoming significant only 30 to 60 minutes after the product is mixed with the blood.

Similar experiments performed with 2-methyl-1,4-naphthoquinone

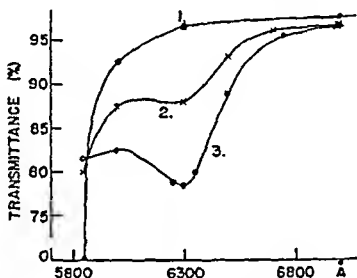


FIG. 3. Relative transmittance of a sample of dog blood at 1:500 dilution (Curve 1), after 30 minutes exposure to 0.1 volume of saline saturated with 2-methyl-1,4-naphthoquinone (Curve 2), and after treatment with ferricyanide (Curve 3).

gave entirely different results. Within 2 minutes after the blood was mixed with solutions of 0.9 per cent saline saturated at room temperatures with the naphthoquinone (119  $\gamma$  per cc.), there was a noticeable appearance of methemoglobin. It was definite at 5 minutes, pronounced at 15 minutes, and increased to the extent shown in Fig. 3 at 30 minutes.

Since the solubility of 2-methyl-1,4-naphthoquinone in water is rather low, it was necessary in these experiments to use 4 to 10 cc. samples of plasma for the analyses. A single large sample of oxalated blood was used, and 2 cc. of saline, saturated at room temperature with the naphthoquinone, were added to 18 cc. portions of the blood. For each determination a control, containing no naphthoquinone, was set up. Samples and their controls were centrifuged 5, 15, and 60 minutes after their preparation and the plasma was tested without protein precipitation. Recoveries of 30, 19, and 20 per cent, respectively, were obtained 5, 15, and 60 minutes after the

preparation of the samples. With blood from a different dog the recoveries after 5 and 15 minutes were 16.5 and 12 per cent. The erythrocytes were laked and a variety of precipitants and extractions were tried in unsuccessful attempts to recover the naphthoquinone or phthiocol.

To determine whether similar phenomena were observed *in vivo*, 50 to 70 mg. of 2-methylnaphthoquinone in acacia suspension were administered by stomach tube to each of six rats. These rats, weighing 200 to 250 gm., were decapitated 1, 2, 3, 4, 5, and 16 hours after the administration of the drug and blood samples were collected with oxalate. Each animal showed a marked methemoglobinemia but direct analysis of the plasma showed no free naphthoquinones.

It appeared that the antihemorrhagic activity of 2-methyl-1,4-naphthoquinone might be destroyed as a result of its interaction with whole blood. Therefore, 1 mg. of the naphthoquinone in 0.5 cc. of 50 per cent alcohol was added to 10 cc. of fresh blood, and 30 minutes after the preparation of the sample, 1 cc. of blood was diluted to 10 cc. with water, and 0.1 cc. of the diluted blood (0.95  $\gamma$  of the naphthoquinone added) was administered by stomach tube to vitamin K-deficient chicks. In a second series, normal saline saturated at room temperatures with the naphthoquinone was added to whole blood to give a concentration of 1.0  $\gamma$  per 0.1 cc. of undiluted blood. This sample was assayed at 1 and 3  $\gamma$  levels. Dr. W. L. Sampson, who conducted the chick assays, advises us that the samples as tested possessed an activity equivalent to less than 0.5  $\gamma$  of 2-methyl-1,4-naphthoquinone.

From these experiments it would appear that the naphthoquinone is rapidly converted to a water-soluble product as a result of its interaction with whole, unlaked blood. There is a concomitant loss of antihemorrhagic activity and a methemoglobin formation which increases with time.

#### SUMMARY

A rapid colorimetric method for the determination of 2-methyl-1,4-naphthoquinone has been devised on the basis of the reaction with cysteine in alkaline solution. The method is directly applicable to the determination of sodium 2-methyl-1,4-naphthoquinone-3-sulfonate.

When added to plasma, 2-methyl-1,4-naphthoquinone is, in part, adsorbed by or undergoes addition reactions with the plasma proteins. Both the free and the conjugated forms of the naphthoquinone undergo an irreversible air oxidation.

When added to whole, unlaked blood, the naphthoquinone produces methemoglobin formation and as much as 70 to 90 per cent of the naphthoquinone is converted to a water-soluble product within 5 minutes after admixture with the blood. This product appears to be devoid of antihemorrhagic activity. Following oral administration of the naphtho-



quinone to rats, there was a marked methemoglobinemia, but the unchanged naphthoquinone could at no time be recovered from the blood.

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# THE ACTION OF PAPAIN ON BEEF SERUM PSEUDOGLOBULIN AND ON DIPHTHERIA ANTITOXIN

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It is generally believed that the proteolytic action of papain differs from that of enzymes of animal origin such as pepsin and trypsin. Annetts (1) found that when egg albumin is treated with papain-cyanide at pH 5 the course of digestion is marked not only by a progressive increase in material of low molecular weight but also by changes in the remaining albumin molecules. From ultracentrifugal and electrophoretic evidence it appeared that no unchanged egg albumin remained in the digests. In contrast to this, Tiselius and Eriksson-Quensel (20) showed that during the digestion of egg albumin by pepsin the remaining heavy protein retains the physical and immunological properties of native egg albumin, even when a considerable fraction of the albumin has been split into fragments of low molecular weight. Lundgren (6) has shown that crystalline papain catalyzes the denaturation of thyroglobulin prior to hydrolytic fissure of the protein. Papain also inactivates typhoid agglutinins which are resistant to pepsin and trypsin (16), and ragweed pollen extracts resistant to pepsin, trypsin, and erepsin (7). Whereas pepsin acts at hydrogen ion concentrations at which its substrate is positively charged, and trypsin on negatively charged proteins (8), papain has its pH optimum at 5.0, near the isoelectric point of most proteins (21).

In view of the destructive action of papain on other proteins, the finding by Pope (14) that active horse diphtheria antitoxin can be recovered from antibody treated with papain, as well as with pepsin and trypsin, is surprising. It seemed of interest to determine whether this protein was unique in its resistance to the enzyme, or whether the action of papain might be less severe than has generally been thought.

An ultracentrifugal analysis of the action of papain on beef serum pseudoglobulin and on diphtheria antitoxin has therefore been made. These proteins have the advantage, as shown previously (11, 12), that their primary and secondary cleavage products are still large enough to retain their protein characteristics, and to sediment easily. In the case of the antitoxin Pope's finding has been confirmed and the sedimentation properties and flocculation behavior of the digests have been studied.

*Materials*—The beef serum pseudoglobulin was prepared by ammonium sulfate fractionation and dialysis, as previously described (11).

The antitoxin was the purified pseudoglobulin, 35 per cent precipitable by toxin, whose splitting by pepsin has been studied in this laboratory (12).

The papain was a commercial preparation, papain, Merck, except in one experiment for which a small amount of the crystalline papain prepared by Balls and Lineweaver (2) was available. These crystals, supplied to this laboratory several years ago through the kindness of Dr. A. K. Balls, had retained only 1 per cent of their activity. Freshly crystallized papain cannot be obtained at the present time.

*Procedure*—The beef pseudoglobulin was digested by the method of Annetts (1). The papain was ground in a mortar with 0.2 M acetate buffer, pH 5.0, and suspended in buffer. Equal amounts of 2 M KCN and 2 M acetic acid were added, and the suspension stoppered tightly and kept at 40° for 2 hours, to activate the enzyme. Pseudoglobulin in acetate buffer, containing about 40 mg. of protein per cc., was then added, and the mixture kept at 40°. The digests contained about 2 per cent protein. After various time intervals samples were withdrawn from the digest and transferred to Visking bags for dialysis against phosphate-borate buffer, pH 7.1, containing 1 per cent sodium chloride. In some experiments the hydrogen cyanide was removed *in vacuo*, but this was found to be unnecessary when the digest and the first change of dialysis buffer were made 0.5 per cent in hydrogen peroxide by the addition of Merck's superoxol, and the buffer was chilled before use. Further details of the procedure are shown in Table I.

The antitoxin was digested as described above, and also by Pope's procedure (14). The ground papain was suspended in water and activated with thioglycolic acid for 30 minutes at room temperature. The insoluble material was then removed by centrifugation and an aliquot of the supernatant adjusted to pH 4.0 with solid sodium bicarbonate. Enough antitoxin was added to make a 1.4 per cent solution, and the pH was adjusted to 4.1 with solid citric acid. The digestion mixture was heated at 50° for 45 or 90 minutes, then chilled. The enzyme was inactivated with hydrogen peroxide and the digest set in the refrigerator overnight. A gelatinous precipitate was removed by centrifugation and the supernatant dialyzed against cold buffer as above.

A portion of the digest was heated at 58° for 30 minutes at pH 4.2 in 5 per cent sodium chloride. Pope (15) has shown that under these conditions the antitoxic fragments formed by peptic digestion at pH 4.2 are soluble, whereas the inactive portion of the molecule is coagulated. The precipitate was removed by centrifugation, and the supernatant dialyzed against the phosphate-borate-chloride buffer.

After dialysis, both heated and unheated solutions were tested for flocculation with purified diphtheria toxin (10).

The non-dialyzable fractions of the digests were analyzed in the Svedberg oil-turbine ultracentrifuge for their molecular mass spectra, as described in a previous paper (11). The Lamm scale method was used to

TABLE I  
*Digestion of Beef Serum Pseudoglobulin by Papain*

Experiment No.	Pseudoglobulin		Activator	Time of digestion	Protein concentration	Per cent total protein left	S <sup>7</sup>		S <sup>8</sup>		S <sup>11</sup>		S <sup>1</sup> , per cent total protein
	mg	mg.					Per cent total protein	S <sub>20</sub>	Per cent total protein	S <sub>20</sub>	Per cent total protein	S <sub>20</sub>	
P1	84	4*	0.04 M KCN	13	0.5					5.3			
P2A	76	3*	0.02 " "	20	1.3	81	23		44	5.3	15		0
					0.7					5.3			
B	76	3*	0.02 " "	40	0.8	47	4		9		32	3.5	2
C	76	3	0.02 " "	63	0.5	34	3		6		25	4.0	0
D	76	3		63	1.4	89	47	7.0	28	4.9†	13	3.1†	0
P3A	172	9*	0.07 M KCN	20	1.0	54	0		13	5.0†	36	3.6	5
B	345	18	0.07 " "	61	0.6	28	0				23	3.5	4
C	172	9	0.3% H <sub>2</sub> O <sub>2</sub>	61	2.0	98	69	6.6	13		17	3.8†	0
P6A	37	2*	0.07 M KCN	$\frac{1}{2}$	1.9	92	80	6.5	3		9		0
B	37	2	0.07 " "	$\frac{1}{2}$	1.9	92	75	6.6	6		10		1
C	32	2	0.07 " "	1	1.9	91	59	6.6	16		13		3
					0.9			6.7					
P4A	179	9*	0.07 " "	3	1.6	79	17		42	4.9	17		3
B	179	9	0.07 " "	6	1.4	69	8		42		17		2
C	179	9	0.07 " "	10	1.3	63	6		31	5.1	21	3.5	5
D	89	4.5	0.07 " "	15	1.1	52	5		19		26		2
E	268	13.5	0.07 " "	23	1.0	49	4		11		32	3.8	2
F	54	2.7	0.07 " "	35	0.9	40	1		6		31		2
G	54	2.7	0.07 " "	55	0.6	28	1		3		23	3.7	1
H	54	2.7	0.07 " "	83	0.6	26	1		2		22		1
P5A	46	2.2†	0.07 " "	2	1.5	78	9		52	4.9	10		7
B	46	2.2	0.07 " "	10	0.8	43	1		14	4.7†	25	3.5†	3
C	59	2.7	0.07 " "	50	0.3	19	1		2		15	3.8	1
Untreated pseudoglobulin					0.9			7.0					
					1.5			6.8					
					2.1			6.4					

\* Crude papain, 1 mg of protein =  $5 \times 10^{-7}$  [Pa. u]<sup>HA</sup>.

† Approximate value.

‡ Crystalline papain, 1 mg of protein =  $2 \times 10^{-7}$  [Pa. u]<sup>HA</sup>.

determine the position of the boundaries in the cell. Line displacement-distance diagrams obtained after about 120 minutes of centrifugation at 60,000 R.P.M. were resolved into their separate components, as indicated

in Fig. 1. The relative amounts of the various components were determined. The sedimentation constants of the predominating components could be measured accurately; those of the minor components are approximate values, and are noted in Tables I and II.

The diffusion constant of one sample of digested pseudoglobulin was measured in a Lamm cell. The boundary spreading was observed by the scale method, and the diffusion constant calculated by the method of moments.

The untreated beef pseudoglobulin and some of the digests were also

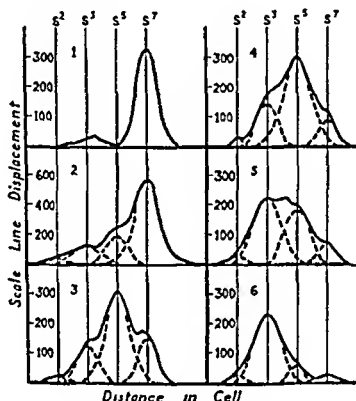


FIG. 1. The digestion of beef pseudoglobulin by crude papain. The broken lines indicate the separate components. Curve 1, untreated pseudoglobulin. A small amount of X component is present. Curve 2, Experiment P6C, 1 hour digestion. Curve 3, Experiment P4A, 3 hours digestion. Curve 4, Experiment P4B, 6 hours digestion. Curve 5, Experiment P4D, 15 hours digestion. Curve 6, Experiment P4G, 55 hours digestion.

examined in the Tisliuss electrophoresis apparatus. Observations were made by means of the Svensson-Philpot schlieren system.

The protein concentrations of all solutions were determined by the micro-Kjeldahl technique. In calculation of the amount of material left after dialysis, correction was made for any changes of volume during dialysis.

The activity of both the crude and the crystalline papain was measured by the hippurylamide method (2). The crude enzyme was also assayed by the milk-clotting method (2). The amounts of papain given in Tables I and II represent only the non-dialyzable soluble protein in the preparations used.

TABLE II  
*Digestion of Horse Diphtheria Antitoxin by Papain*

Experiment No.	Anti-toxin mg.	Papain* mg.	Activator	Temperature of digestion °C.	Time of digestion hrs.	Heated at 53° pH 4.2, 5 per cent NaCl	Protein concentration per cent	Per cent total protein left	s <sup>2</sup> per cent total protein	s <sup>3</sup>		s <sup>2</sup> per cent total protein	Lf units per mg.
										Per cent total protein	f <sub>20</sub>		
AP <sub>1</sub>	70	9	Thioglycolic acid	• 50	‡	Unheated	1.0	70	0	37	5.8	23	33
			"	• 50	1½	Heated	0.3	35	0	16	6.2	19	33
	140	18	"	40	20	Unheated	1.0	32	2	11	5.6	20	25
AA <sub>1</sub>	140	7	0.07 M KCN	40	20	"	1.6	49	0	26	5.9	18	31
						Heated	0.85	26	0		5.5		30
						Supernatant	0.83	37	0	12		21	
AA <sub>2</sub> <sup>a</sup>	57	2.7	"	40	4	Unheated	1.8	86	9	63		14	
	57	2.7	"	40	6	"	1.6	71	4	55	5.3	12	48
	70	3.3	"	40	10	"	1.5	73	4	57	5.3	12	
	c <sub>2</sub>					Heated	0.77	37	0	28	5.4	9	60

\* Crude papain, 1 mg. of protein =  $5 \times 10^{-7}$  [Pa. u.] HA.

† 1 Lf unit is equivalent to 0.00046 mg. of toxin nitrogen.

‡ Approximate value.

### Results

The results from the experiments on beef serum pseudoglobulin are given in Table I. For convenience the various components which appear in the sedimentation diagrams have been named after their sedimentation constants. Thus the  $s^7$  component has  $s_{20} = 7 \times 10^{-13}$  cm. per second per unit of force, or 7 S. (svedbergs). The  $s^7$  component is the unsplit globulin, and  $s^5$ ,  $s^3$ , and  $s^2$  are the non-dialyzable fragments formed from it. The small peak due to the papain is hidden in the  $s^3$  peak. From Experiments P6 and P4, Table I, it may be seen that the amount of unsplit globulin decreases rapidly with the time of digestion. The first split-product, the  $s^5$  component, increases in amount but later is further broken down into the  $s^3$  and  $s^2$  components, and then into fragments small enough to dialyze through cellophane. Representative line displacement-distance diagrams are shown in Fig. 1.

Similar results were obtained in Experiment P5, when crystalline papain was used. After 2 hours digestion, the size distribution resembled that in Curve 4, Fig. 1; after 10 hours, that in Curve 5; and after 50 hours, that in Curve 6.

The sedimentation constants found for the unsplit globulin and its various digestion products are given in Table I. From these it can be seen that the splitting of the protein proceeds in an orderly and reproducible fashion. There is no evidence of "denaturation." Although the amount of unsplit protein decreases rapidly, its sedimentation characteristics remain the same. The variations in sedimentation constant with protein concentration, emphasized by Kabat in a careful study of the molecular weight of antibodies (4), are the same for the unsplit protein left in the digests as for the original pseudoglobulin. The greater spreading of the  $s^7$  peak in Curve 2 is in proportion to its greater height. The ordinates were reduced to one-half when this curve was plotted.

The  $s^5$  component has a sedimentation constant of 5.3 S. in dilute solution. This is similar to 5.4 S., the value found for the first split-product in peptic digestion (11). Since, as shown in Curves 3 and 4, Fig. 1, the  $s^5$  boundary is as sharp as that of a homogeneous protein, the first split-products must be approximately equal in size. The sedimentation constant, 5.3 S., is about what would be expected for fragments of 90,000 molecular weight, halves of the original pseudoglobulin. Since, in Experiment P4A, 42 per cent of the protein is in the  $s^5$  component, at a time when only 21 per cent has been lost, the possibility that the half molecules have been formed by the breaking off of small fragments must be ruled out; a splitting of the molecule in a plane normal to the long axis, as previously suggested (11), explains the results most satisfactorily.

For the  $s^3$  component the average sedimentation constant is 3.7 S. As is shown in Fig. 1, Curve 6, this component may be obtained relatively free of larger molecules, after sufficiently long digestion. The diffusion constant of this digest (Experiment P4G) was found to be  $7.5 \times 10^{-7}$  sq. cm. per second. Because of the inhomogeneity of the material this value is only an approximate one; but it does indicate that the  $s^3$  component consists of fragments of about 45,000 molecular weight, quarters of the original pseudoglobulin molecule.

The small amounts of the  $s^2$  component present indicate either that these fragments are small enough so that most of the component is lost on dialysis or that they are rapidly broken down into dialyzable fragments. The sedimentation constant of this material was not measured.

Two control experiments were performed. In the first of these, Experiment P2D in Table I, no cyanide was added before the 2 hour activation period. Enough active enzyme was present, however, to split half the protein in 63 hours. The line displacement-distance diagram resembled Curve 2 in Fig. 1. The unactivated enzyme also showed some activity in the milk-clotting test, 0.16 unit per mg. of protein nitrogen, as compared with 2.4 units per mg. for the activated enzyme. In a second control experiment, Experiment P3C in Table I, the enzyme was incubated for 2 hours at  $40^\circ$  with 0.3 per cent hydrogen peroxide. A very small amount of active enzyme remained even after this treatment, and some splitting of the pseudoglobulin subsequently took place. The unsplit protein, however, retained the sedimentation characteristics of the original pseudoglobulin, indicating that no denaturation had taken place under the conditions of digestion in 61 hours.

The pseudoglobulin and two of the digests were examined in the Tiselius electrophoresis apparatus in phosphate-borate buffer, pH 8.05, ionic strength 0.10. The pseudoglobulin showed one broad but symmetrical peak, with a mobility of  $-2.2^1$  and a faster component with a mobility of  $-5.2^1$  comprising about 10 per cent of the total. After digestion for 61 hours (Experiment P3B) the globulin showed a symmetrical peak with about the same amount of spreading, but a mobility of  $-1.2^1$ . Pseudoglobulin digested for 61 hours with inactivated papain (Experiment P3C) showed one broad asymmetrical peak. The faster component had disappeared, and the remaining material had an average mobility of about  $-3.0$ . It appeared to be a mixture of unchanged pseudoglobulin and some slightly faster moving material. No separation could be obtained, however. The rapid spreading of the globulin, and the small changes in mobility caused by digestion, make electrophoretic patterns of these

<sup>1</sup> Electrophoretic mobilities are expressed in sq. cm. per second per volt  $\times 10^5$ .



digests difficult to interpret, so no more electrophoresis experiments were attempted.

*Antitoxin*—Horse diphtheria antitoxin digested by Pope's method still flocculated with toxin. After 45 minutes digestion (Experiment AP<sub>1</sub>, Table II) the non-dialyzable protein left showed components with sedimentation constants of 5.8 and 4.0 S. The protein not precipitated by heating at 58° also flocculated with toxin, and its sedimentation constants were about the same. After 1½ hours digestion (Experiment AP<sub>2</sub>) much more proteolysis had taken place, but the sedimentation constants of the components were about the same, and active antitoxin was still present.

Digestion by Annetts' method, however, gave much better results. The antitoxic split-products had sedimentation constants of 5.3 to 5.9 S.

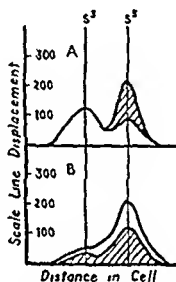


FIG. 2. Horse diphtheria antitoxin digested by crude papain. The upper curve in each section was obtained on the total digest. (A) The shaded portion = protein precipitated by toxin (Experiment AA<sub>1</sub>); (B) the shaded portion = protein soluble at 58° (Experiment AA<sub>2c</sub>).

and 3.4 S., as shown in Table II. The antitoxin in Experiment AA<sub>1</sub> was 22.5 per cent precipitable by toxin, as compared with 35 per cent precipitable before digestion. Fig. 2, A shows the line displacement-distance diagrams for this digest. The clear area represents the supernatant after precipitation of the antitoxin with purified toxin; the scale is corrected for the dilution due to toxin addition. The upper curve is that obtained on the total digest, and the shaded area therefore represents the precipitated antitoxin. No loss of the s<sup>3</sup> component is apparent. Fig. 2, B shows the line displacement-distance diagrams obtained in Experiment AA<sub>2c</sub>. Digestion has proceeded for a shorter time, and so a smaller amount of component s<sup>3</sup> is present. Here the shaded area represents the protein not precipitated on heating at 58°.

From these experiments it appears that the digestion of horse antitoxin by papain is analogous to its splitting by pepsin (12, 19) and trypsin

(9, 17). The molecule is split into approximate halves, one of which carries the antitoxic activity and is soluble at 58°. These fragments are then split again, into quarters. None of the quarters is precipitable by toxin; but some of them, presumably from the antitoxin halves, are soluble at 58°.

#### DISCUSSION

Ultracentrifugal analysis thus provides evidence that papain splits beef serum pseudoglobulin and horse diphtheria antitoxin in an orderly and reproducible fashion, into halves, quarters, and dialyzable fragments. There is no evidence of "denaturation," and the antitoxin halves resemble closely those obtained on peptic digestion, in solubility and precipitability by toxin as well as in size.

In an interesting study of the action of crystalline papain Lineweaver and Hoover (5) showed that, although papain attacks native hemoglobin much less rapidly than it does denatured hemoglobin, it does digest the native protein. As further proof of the ability of proteolytic enzymes to act on native proteins they cite the formation of trypsin, pepsin, and chymotrypsin from inactive precursors. The retention of activity when antibodies, such as diphtheria and tetanus antitoxins (15, 18) and pneumococcus anticarbohydrate (3, 13), are split by proteolytic enzymes is further evidence for this hypothesis.

The kinetics of a process such as the enzymatic breakdown of a protein must be very complex, with primary, secondary, tertiary, and so forth cleavages proceeding at different rates. Although the amount of enzyme present is constant, the number of substrate particles competing for it is increasing, so that only with a great excess of enzyme would first order reactions be possible. From Table I it may be seen that the rate of disappearance of the  $s_7$  component is much more rapid than is the loss of non-dialyzable protein. If, in a study of the effect of proteolysis on a serum globulin, the loss of some function that depends on the intact molecule is compared with the decrease in total protein, or increase in non-protein nitrogen, no parallelism in rate can be expected. If the function under observation is also a property of half molecules, it will be relatively more resistant to proteolysis; this is particularly true of the antitoxins and pneumococcus anticarbohydrates. Substances such as the typhoid agglutinins studied by Rosenheim (16), which became resistant to the action of pepsin and trypsin but not to that of papain, may differ in resistance to primary or secondary cleavage. In view of the complexity of the processes involved, criteria of the extent of proteolysis should be interpreted with great caution.

## SUMMARY

1. Beef serum pseudoglobulin is split by papain into fragments with sedimentation constants of 5.3 and 3.7 svedbergs, corresponding to halves and quarters of the original molecule. The quarter molecules are further split into fragments small enough to dialyze through cellophane.

2. Although the amount of intact globulin decreases rapidly as digestion proceeds, its sedimentation characteristics remain unchanged, indicating that no denaturation has taken place.

3. Crystalline papain and crude papain split the globulin in the same way.

4. Horse diphtheria antitoxin is split by papain into equal fragments, only one of which flocculates with toxin and is soluble at 58° at pH 4.2 in the presence of 5 per cent sodium chloride. These fragments have a sedimentation constant of about 5.5 S., and appear to be approximately halves of the original molecule. These halves are further split into fragments of sedimentation constant 3.4 S., probably quarters of the original globulin.

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# DISTRIBUTIONS OF ESTROGENS BETWEEN IMMISCIBLE SOLVENTS\*

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In the development of quantitative methods for the extraction and estimation of small quantities of urinary steroids an investigation into certain physical properties of the pure steroids became necessary. Exact data are few and have appeared only recently. Modern estimations of solubility of the estrogens were reported by Doisy *et al.* (1). Concerning the distribution of pure steroid hormones between immiscible solvents, only scattered reports appear in the literature. At the time of the preliminary report of the present paper (2), similar work on the solvent distributions of the estrogens had been initiated in another laboratory, and Bachman and Pettit have since reported their results (3).

*Estimation of Estrogen*—Practically all of the results to be reported were obtained by the use of bioassays, and colorimetric methods were used merely to confirm some of the results. The obvious advantages of color assay and the known difficulties arising from bioassay do not outweigh the fact that in any experimental approach the estimation of estrogenic compounds must ultimately be referred to their biological activity. Not only are the current color methods non-specific for any single individual among growing series of naturally occurring steroids, but the lability of many of the active compounds to physical treatment, as shown by the losses of physiological activity, can be controlled at present only by following biological potency.

An important factor in the prevention of destruction is the elimination by bioassay of the additional manipulations necessary for purification of extracts for color assay, and in many instances the low concentrations of estrogen require the more sensitive bioassay procedures.

*Bioassay*—An adequate assay of mixed estrogen extracts of low potency requires ideally that the animal should be small (for greatest sensitivity), should belong to a highly uniform stock, and should be capable of being reused quite often over fairly long periods; the potency of all active compounds should be of the same order and show qualitatively the same physiological reaction for the test procedure selected. The latter conditions are necessary for even an approximate estimation of total estrogen in extracts

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containing estriol in order that the value be somewhere near that of the sum of the separate potencies of each estrogen. The biological action of estriol varies considerably with the test procedure used, and its low activity is attributable chiefly to its slower reaction. When administered with sub-minimal amounts of other estrogen, estriol may show a considerable "X substance" effect. Activity of the triol may be greatly increased by lengthening the period of administration, as shown in Table I. The use of injections in oil does not eliminate the necessity for a longer course of administration in mice.<sup>1</sup> Oil media have other disadvantages which reduce the usefulness of the assay animal, such as slow and variable absorption, encapsulation of injection areas which may release active material upon subsequent injection, and longer periods of recuperation between assays.

TABLE I  
*Biological Activities of Estrogens by Aqueous Assay Procedures*

Assay procedure	Estrone	Estriol	$\alpha$ -Estradiol
	$\gamma$ per unit	$\gamma$ per unit	$\gamma$ per unit
Doisy rat unit* (3 aqueous injections, 4½ hrs. apart)	0.60	0.7-1.0	0.06
Doisy mouse unit (same procedure as above)	0.04	>2	0.02
Marrian mouse unit (4 aqueous injections, 12 hrs. apart)	0.035	0.10	0.015
Modified Marrian mouse unit (4 aqueous injections at 0, 9, 24, and 33 hrs.)	0.035	0.11	0.015

\* Values furnished by Dr. S. A. Thayer. The mouse assay values checked well with Dr. Thayer's averages obtained by standardizing assays in this colony.

For these reasons assays were conducted by a modification of the Marrian procedure which gave essentially the same biological activities for crystalline estrone, estriol, and  $\alpha$ -estradiol as the original. Extracts were taken up in dilute alkali (0.001 N NaOH) and the pH adjusted to approximately 9 with phenolphthalein, the final volume being such as to contain approximately 1 unit in 2 ml. Each dose was divided into four injections which were administered subcutaneously into spayed mice at 8.00 a.m. and 5.00 p.m. on consecutive days. Smears of vaginal epithelium were made with moist cotton swabs on the evening of the 3rd day, morning and evening of the 4th day, and morning of the 5th day of assay, and evaluation of the results followed the recommendations of Doisy that leukocytes be entirely replaced by epithelial cells, with a majority of the cells keratinized. A five point grading system was used merely for reference, the

<sup>1</sup> Emmens (4) has reported extensive observations of these relationships.

two highest grades, corresponding to majority and total keratinization, being considered positive.

Owing to the many assays demanded and to the limited amounts of extract available in many instances, the pharmacological method of response curve readings from single dose administration to a large group of animals was replaced by a method of graded dosage. Except for the standardizing assays of stock solutions and final assays of critical fractions, groups of only three or four animals were assayed at any one dosage. For completely unknown titers a wide range was covered by four or five groups; for narrower ranges one group was assayed at the expected level, and one group each above and below that value. From the preliminary assays the approximate level could usually be estimated, and final assays were run at the determined level and at 10 per cent above and below (1.8, 2.0, and 2.2 ml. total), larger groups being used whenever possible. For a positive assay a plurality of positive responses in any dosage group was required.

Since the complete assay of each preparation was spread over several assay periods (assays were begun twice each week) day-to-day variation in sensitivity of the colony was controlled in some measure, and conflicting results could be checked by another reassay. The over-all results corresponded closely to those for the 50 per cent response at the test level required by the original Marrian procedure, as confirmed by the assay of standard stock solutions. The values of the animal units for the standardized St. Louis colony are given in Table I.

*Colorimetric Assay*—The color reaction used for the estrogens is a sulfuric acid method to be described more fully in a later report (5). While with proper control this or any other method of the Kober type is quite capable of reproducible results with pure hormone solutions, a certain amount of the steroid undergoes chemical change during even mild physical manipulation, and highly chromogenic degradation products may be concentrated in the fraction containing the least estrogen. The estimates of steroid by non-specific color methods in such a case may easily vary from those by bioassay; other contributing factors have been mentioned above. In the determination of distribution ratios of extreme value, say, 10:1 or 1:10, the values obtained for chromogenic steroid in the less concentrated fraction may quite easily vary from those for estrogen by bioassay in the direction of erroneous high values.

*Distribution of Crystalline Estrogens*—Crystalline estrogens of high quality<sup>2</sup> were used in amounts which were safely within the limits of solu-

<sup>2</sup> Kindly furnished by Professor E. A. Doisy. The estrone and estriol samples had been repeatedly recrystallized to constant melting point; the  $\alpha$ -estradiol was obtained by catalytic reduction of estrone, and purified by separation with Girard's reagent.

bility of both solvents, and although the solubilities of estriol in several solvents were liminal, its alkaline solubilities were sufficient to prevent overloading of the mole fractions for the organic solvents.

10 ml. aliquots of standardized alcoholic estrogen solutions were evaporated and taken up, usually in the alkaline portion, 100 ml. volumes of each solvent at 20° being used. The quantities of estriol did not exceed 1.1 mg., of estrone 0.46 mg., and of  $\alpha$ -estradiol 0.18 mg. The samples were distributed by 10 minute equilibrations in separatory funnels. The organic solvent was distilled off, the aqueous fraction was acidified and extracted with ethyl ether, and each residue taken up in dilute alkali, neutralized, and appropriately diluted for assay.

Special precautions were taken to insure as complete removal as possible of the more concentrated fractions from the mixtures, and to limit destruction by neutralization and the removal of residual organic solvent in the alkaline fraction, and by buffering the organic fractions with acetate before distillation. Despite such care, distributions between normal alkali and several solvents gave evidence of appreciable loss in the total recoveries of biological activity, and several values with this concentration of alkali are therefore undeterminable by either type of assay.

The results may be expressed either as ratios or as percentage coefficients, and for simplicity are reported as average coefficients of the distributions into the organic phase. These values were usually calculated from the ratios of assay values of organic to aqueous fractions for only those partitions in which total recoveries were greater than 85 per cent; for extreme distributions into either solvent the coefficient was calculated from the least concentrated fraction and the original total, since the assay error was multiplied by a factor of from 5000 to 10,000.

The degree of accuracy of the coefficients presented in Table II is difficult to estimate. While with a number of distributions the variability among trials was rather small, with certain solvents, notably the ethers, greater variability is unavoidable. In general the extreme ratios were surprisingly reproducible in view of the sources of error, although many are so one-sided as to be of relative value only (e.g., a comparison of the ratios of 700:1 for estrone with 200:1 for estriol in the butanol-carbonate partitions). However, with extreme coefficients the degree of completeness of the separation of solvents cannot be standardized, and in their use the results will be affected by the same factor. Percentage values carried into decimals, therefore, represent merely the closest estimate obtainable. It should be pointed out, parenthetically, that, whereas for values in the mid-range an error of  $\pm 10$  per cent represents a considerable quantity of estrogen in terms of mouse units, the considerably greater error in a determination of 99.5 per cent in the organic phase may be negligible for that phase and

quite important for the aqueous phase; the decimals are therefore significant in spite of the greater error, and may be used to advantage in quantitative calculations.

With the exceptions noted, the values have preferentially been based

TABLE II  
*Distribution Coefficients of Estrogens between Immiscible Solvents*

Organic solvent	Aqueous solvent	Per cent estrogen in organic phase		
		Estrone	Estriol	$\alpha$ -Estradiol
Ethyl ether	Water		86	
	0.3 M $\text{Na}_2\text{CO}_3$	99.5	65 (Ca.)	99.7
	0.1 " NaOH	50	2	70
	1.0 " "	30		30
<i>n</i> -Butanol	0.3 " $\text{Na}_2\text{CO}_3$	99.9	99.5	>99.7
	0.1 " NaOH	90	85*	97
	1.0 " "	*	*	98*
Benzene	0.1 " HCl	>99.7	14	
	0.3 " $\text{Na}_2\text{CO}_3$	99.6	2.5	95
	0.1 " NaOH	60	0.3	17
	1.0 " "	20		5
Isopropyl ether	0.3 " $\text{Na}_2\text{CO}_3$	99.5	50 (Ca.)	99.5
	0.1 " NaOH	50	2	65
	1.0 " "			23
Toluene	0.3 " $\text{Na}_2\text{CO}_3$	99.6	2	97
	0.1 " NaOH	50	0.3	80
	0.1 " HCl		25	
Carbon tetrachloride	0.3 " $\text{Na}_2\text{CO}_3$		6.5†	50†
	0.1 " NaOH	15†	<2.5†	5†
Chloroform	0.3 " $\text{Na}_2\text{CO}_3$	99.7	20	99
	0.1 " NaOH	92	1	50
	1.0 " "			5
Petroleum ether (35-52°)	0.3 " $\text{Na}_2\text{CO}_3$	15 (Ca.)	0.2	5
	0.1 " NaOH			0.5
Dibutyl ether	0.3 " $\text{Na}_2\text{CO}_3$	97†	5 (Ca.)	80 (Ca.)
	0.1 " NaOH		1 "	45 "

\* Destruction of biological potency; where substituted for the value, less than 85 per cent of total potency was recoverable for any trial.

† Values reported for colorimetric assays only.

upon the results of bioassay, and for simplicity the duplicating colorimetric averages are not listed.

*Separation of Estriol from Phenolic Fractions*—Based upon the distributions in benzene, the following procedure has been used for some time in separating estriol from pure estrogen mixtures or from urinary extracts. It may be applied directly to the dried residue of crude ether extracts of



urine, although the triol (carbonate) fraction contains the bulk of the non-steroid impurities and must be assayed biologically at this stage.

The residue of the total estrogen fraction to be separated is taken up in a convenient volume (50 to 100 ml.) of 0.3 M sodium carbonate and extracted with an equal volume of benzene. Each fraction is reextracted with equal volumes of the opposite solvent and the two washes are then extracted together. Benzene and carbonate fractions are both collected, the former containing the neutral estrogen (and other steroid if not previously separated) and the latter the estriol. The triol may be removed from the carbonate with considerable purification by sufficient ether extraction without neutralization, but with bioassay procedures may either be assayed directly or concentrated by neutralization to pH 9 and ether extraction. The theoretical separation is 99.5 per cent; this may be attained in practice by careful manipulation by virtue of the washing out effect of the double extraction, since the theoretical coefficients are limited by the impossibility of complete separation.

#### DISCUSSION

A comparison of the distribution data with those recently reported for solubility (2) reveals little correlation of the two properties among the several solvents for any one compound, and almost no relationship for any one solvent with the several estrogens. Roughly speaking, of those solvents examined, butanol shows the greatest solvent power, the petroleum fractions the least.

Several useful conclusions may be drawn from the results.

The problem of extraction and treatment of the estrogen fraction as a whole is considerably hampered by the divergence of estriol distributions and solubilities from those of the group, which might include the equilins. Care must be exercised to prevent its loss during any manipulation.

Except for requirements of large scale extractions which might profitably utilize the extreme coefficients of *n*-butanol, the lower ethers prove to be preferable as extractants of total estrogen. While both present problems in peroxide formation, the greater volatility and inflammability of ethyl ether give isopropyl ether an appreciable advantage.

*n*-Butyl ether is not particularly useful in extractions of aqueous media.

Their sharp differentiation between estriol and the other two estrogens renders benzene and toluene useless for extraction of total estrogen, but permits a quantitative separation of the triol from mixed extracts.

The petroleum ethers, notorious for their selective lipid solubilities, are poor solvents for the phenolic steroids and for most of the urinary chromogens, excellent solvents for non-phenolic steroid from even strong alkali (unpublished data). They may therefore be used to clear interfering

steroid (androgen, etc.) from estrogen extracts being prepared for colorimetric estimation. Possibly of greater potential importance is the application in this laboratory of these distributions to the extraction from urines of a steroid fraction, free of estrogen and of practically all color, containing the total free androgen fraction. Of several comparable values these are the only ones which do not agree with those of Bachman and Pettit.

The less inflammable solvents prove unfortunately to have limited applicability.

The comparatively strong acidic nature of estriol, as illustrated by its extreme solubility in pyridine (2), may account for much of its behavior in alkaline distributions, but its considerable solubility in water must contribute to the unexpected partition from benzene into dilute acid.

#### SUMMARY

Distribution coefficients for estriol, estrone, and  $\alpha$ -estradiol between immiscible solvents are reported, and their application to extraction problems indicated. While the less volatile solvents may be valuable in certain applications, diethyl and diisopropyl ethers are recommended for the extraction of total estrogen. The distributions between benzene and 0.3 M sodium carbonate solution are utilized to separate estriol from estrogen fractions, and those between petroleum ethers and 0.1 M NaOH to separate the estrogens from androgen and other interfering steroid.

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# CHLOROFUCINE (CHLOROPHYLL $\gamma$ ), A GREEN PIGMENT OF DIATOMS AND BROWN ALGAE

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Diatoms are the most abundant autotrophic organisms over much of the earth's surface. They and the similarly pigmented brown algae produce prodigious quantities of organic matter. Synthesis of this carbonaceous material, so essential to life in the sea and probably to the formation of petroleum, depends upon absorption of light by pigments in the living algal cells. The number and nature of the green pigments contained in these plants are, however, matters of controversy.

Chlorophyll *a* occurs in all the diatoms and brown algae examined thus far (1-16), but there is no unanimity of opinion regarding the nature and origin of another green substance observed in extracts of these organisms. This second green pigment, originally called chlorofucine (2), later chlorophyllin  $\gamma$  (5, 6), chlorophyll  $\gamma$  (7), and chlorophyll *c* (8), was at first considered a natural constituent of these algae (1-6). Later, from results obtained through the use of an involved analytical method, Willstätter and Page (7) concluded that chlorofucine was a postmortem product. With the exception of Wilschke ((8) p. 355), subsequent workers accepted this conclusion as authoritative even though several of them observed chlorofucine in the extracts of fresh algal material (9-11). Chlorofucine was not observed in land plants (2). It should not be confused with a controversial "chlorophyll *c*" of higher plants (17) which is now believed to have been a mixture of chlorophyll *a* and pheophytin *a* (18).

The occurrence of small amounts of chlorophyll *b* in brown algae was reported by Willstätter and Page (7). There is also one recent report of the occurrence of this pigment in diatoms (16). Chlorophyll *b* has not been detected by other workers who applied various analytical methods to diverse algal species in both these groups (1-6, 8-11, 13, 14). The results of the various pigment investigations are presented briefly in Table I.

In view of the contradictory conclusions just reviewed, it seemed desirable to reexamine the green pigments both of diatoms and of brown algae. Knowledge gained from this reexamination should aid in the interpretation of measurements of photosynthesis in these organisms (14, 15).

TABLE I  
Reported Occurrence of Chlorophyll *b* and Chlorofucine in Various Algae

Bibliographic reference No.	Author	Year	Method	Class of algae*	Chlorophyll <i>b</i>	Chlorofucine
1	Stokes	1864		B.	—	+
2	Sorby	1873	Partition; spectral absorption	"	—	+
3	Reinke	1876	" "	" D.?	—	+
4	MaeMunn	1885	Spectral absorption of extract	"	?	+
5	Tswett	1905	Partition; spectral absorption	" D.?	—	+
6	"	1906	" "	"	—	+
7	Willstätter, Page	1914	HCl alteration	"	$0.05 \times a$	—
8	Wilschke	1914	Fluorescence of extracts	" Y., D.	—	+
9	Kylin	1927	Partition; capillary adsorption	" D.	—	—(?)
10	Bacharach, Dhéré	1931	Fluorescence of extracts	D.	—	+
11	Dhéré, Fontaine	1931	" " "	B.	—	+
12	" Raffy	1935	" " tissue	"	—	—
13	Scybold, Egle	1938	Chromatographic adsorption	" D.	—	—
14	Montfort	1940	" "	" "	—	—
15	Dutton, Manning	1941	" "	D.	?	—
16	Pace	1941	" "	"	$0.1 \times a$	—

\* B., brown (Phaeophyceae); D., diatom (Bacillariophyceae); Y., yellow (Chrysophyceae).

#### EXPERIMENTAL

*Plant Material*—The pennate diatom *Nitzschia closterium* was grown in pure culture by a procedure similar to one previously described (15), except that "snow-white" fluorescent lamps were used instead of a neon lamp. 1 liter of culture usually yielded 0.5 to 1.0 ml. of centrifuged cells. Brown algae were collected at low tide at Moss Beach, north of Half Moon Bay, California. They were identified by Dr. Gilbert M. Smith of Stanford University. Specimens that were not used immediately were kept indoors in shallow open dishes of sea water at a temperature not higher than 17°.

*Methods*—Three general methods have been used for analysis of the chlorophylls in extracts of the algae. The principles of the methods are summarized here. Essential details are described in the sections pertaining to preparation of the individual compounds.

By partition of the pigments between immiscible solvents such as petroleum ether and 80 to 90 per cent methanol, it was possible to separate

chlorophyll *a* and carotene from the xanthophylls and chlorofucine. After dilution of the methanol fraction to 50 per cent with water, most of the xanthophylls were removed from the alcohol-soluble chlorofucine by repeated extraction with ether.

Numerous modifications of the chromatographic adsorption method have been tested for the preparation of chlorophylls. In general the best results were obtained with columns of confectioner's powdered sugar which contained 3 per cent corn-starch to prevent caking. Before use, this sugar was dried at 85° for several hours and was then cooled in closed bottles. In packing the columns, successive small portions of sugar were pressed firmly into the adsorption tube (usually 3 × 30 cm.) with a packing plunger slightly smaller than the tube ((19) p. 42). The filtration rate of columns packed with the "C and H" (California and Hawaiian) brand was nearly twice that of columns of the "Sea Island" (Western Sugar Refinery) brand, although both brands were labeled "Grade XXXXXX." Results nearly as satisfactory were obtained with inulin or with some preparations of starch as adsorbents.

A photoelectric spectrophotometer (20) was used for determination of the spectral absorption of pigments in the extracts of plant material, and for the identification of pigments prepared by partition and chromatographic adsorption methods. Most of the results are presented as the so called characteristic absorption curves, the plot of  $\log \log(I_0/I)$  vs. wavelength (21). For a pure pigment, characteristic absorption curves measured at any concentration are superposable. Variations in the shape of the curves, which are readily observable by superposition, indicate contamination of the pigment with other colored substances. This method of presentation also simplifies the comparison of curves when absolute absorption coefficients are not available. Absorption curves plotted as the  $\log(I_0/I)$  are illustrated by small insets in Figs. 1, 2, and 11.

Extraction of pigments from diatoms was incomplete when acetone (absolute or 80 per cent) or ethanol (absolute or 95 per cent) was used as solvent. The pigments were removed rapidly and completely with absolute methanol. Consequently, this solvent was used for determination of the spectral absorption curves of the algal extracts.

*Chlorophylls a and b from Sunflower*—For standards of comparison it was necessary to obtain spectral absorption curves of chlorophylls *a* and *b* in methanol. These pigments were prepared from sunflower leaves by the partition and chromatographic adsorption procedures employed with algal extracts. About 2 gm. of fresh leaves were extracted rapidly at 20° with 100 ml. of absolute methanol containing about 0.5 per cent redistilled dimethylaniline. (The dimethylaniline served to neutralize plant acids during extraction, to counteract acidity which might otherwise develop

upon the adsorption columns ((19) p. 123), and to minimize pigment oxidation.) Petroleum ether (100 ml.) was added to the extract, followed by about 15 ml. of water. After separation of the methanol layer, the petroleum ether phase was extracted twice with 50 ml. portions of 90 per cent methanol in order to remove the remaining xanthophylls. Residual methanol was removed from the petroleum ether with water, and the green solution was concentrated to a few ml. at reduced pressure and 20°. This concentrated solution was poured onto a column of sugar ( $3 \times 15$  or 20 cm.) which was then washed with small portions of petroleum ether containing 0.5 per cent dimethylaniline. As soon as the carotene had been washed

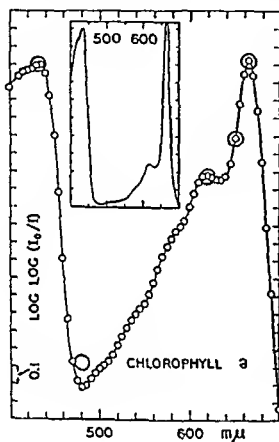


FIG. 1

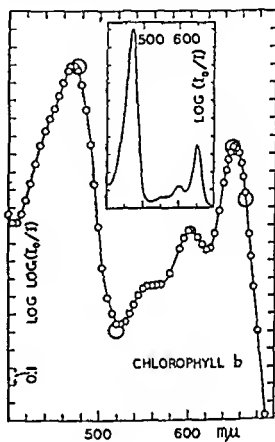


FIG. 2

FIG. 1. Absorption curve of chlorophyll *a*. The inset shows the same data plotted as  $\log (I_0/I)$ . Solvent, methanol. The large circles represent values calculated from Mackinney's results (22).

FIG. 2. Absorption curve of chlorophyll *b*. Solvent, methanol. The large circles are from Mackinney's data (22).

below the chlorophyll, the chromatogram was developed with petroleum ether containing 0.5 per cent dimethylaniline and 0.5 per cent methanol or *n*-propanol. Chlorophyll *a*, which was carried rapidly through the column, was collected separately in the percolate. The yellow-green chlorophyll *b* band moved slowly down the column. This band was removed with a spatula, and the pigment was eluted with freshly distilled ether (U.S.P.). The solution of each chlorophyll was then evaporated nearly to dryness at reduced pressure (20 to 30 mm.). To each residue was added the solvent in which the spectral absorption was to be measured. The remaining ether was removed in a vacuum, and the solutions were then diluted to the

proper concentrations for absorption measurements. The entire preparation required about 1 hour. Consistent spectral absorption curves were usually obtained.

Absorption curves of chlorophylls *a* and *b* in methanol (Figs. 1 and 2) were in satisfactory agreement with values previously determined at a few wave-lengths (22). The absorption by chlorophyll *a* in methanol was quite different from the absorption in ether. The differences were due to solvent effects, rather than to irreversible pigment alteration, because an absorption spectrum of the pigment determined in ether after prior determination in methanol showed the spectral curve typical of ether solutions.

Because of the great lability of the chlorophylls, great care had to be exercised in handling the plant material and also the extracted pigments. If too little alcohol was used for extraction of the pigments, if extraction took too long, or if the chlorophyll remained on the adsorption columns very long, especially in the absence of dimethylaniline and alcohol, additional colored zones were observed on the sugar. In such cases, spectral curves of the recovered chlorophylls exhibited considerable variation in the region between 470 and 600  $m\mu$ . If fresh plant material was permitted to stand with dilute alcohol for a day or more, as is often done in the estimation of chlorophyllase activity (23), and if the pigments were then extracted and adsorbed on columns of sugar, as many as fifteen distinct green bands were observed.

### Results

*Chlorophyll a*—Chlorophyll *a* prepared from *Nitzschia closterium* by adsorption agreed in spectral properties, and in adsorption behavior (*cf.* (19) p. 12), with chlorophyll *a* from sunflower leaves (Fig. 3). Moreover, in the red region of the spectrum, where the presence of small amounts of yellow pigment did not interfere, the curve for chlorophyll *a* prepared from *Nitzschia closterium* by partition between petroleum ether and 80 to 90 per cent methanol was in good agreement with curves for preparations obtained by adsorption (Fig. 3). Since most of the green pigment remained in the petroleum ether, the results prove that chlorophyll *a* is the principal green pigment in the organism. Had chlorophyll *b* been present in appreciable quantities, it would have appeared with chlorophyll *a* in the partitioned extract. Incidentally, Fig. 3 indicates that the adsorption procedure described above did not alter the spectral properties of chlorophyll *a*.

*Natural Occurrence of Chlorofucine*—Fig. 4 shows for the red region of the spectrum a typical absorption curve for a methanol extract of *Nitzschia closterium*. Although the position of the principal absorption maximum and the shape of the curve indicate a large proportion of chlorophyll *a* in the extract, the curve for the extract differs significantly from the chloro-



phyll  $a$  curve. Similar differences were observed when acetone extracts were examined.

Various methods of extraction, some of which had been employed by Wilschke (8), had little or no effect on the shape of the curve for total pigment absorption, as illustrated in Fig. 5. In one case, diatoms from 1 liter of culture were centrifuged, suspended in 1 ml. of water, placed in a boiling water bath, and diluted with 15 ml. of boiling water. After 1.5 minutes the suspension, which had turned a bright green, was cooled quickly with ice, centrifuged, and the cells extracted with methanol. A similar quantity of the same culture, suspended in 1 ml. of water, was shaken in a current of hydrogen for 10 minutes and then extracted with methanol in the stream

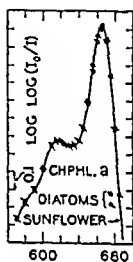


FIG. 3

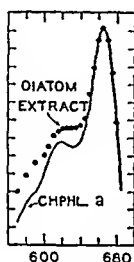


FIG. 4

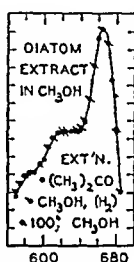


FIG. 5

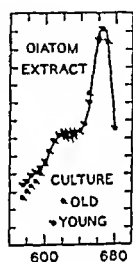


FIG. 6

FIG. 3. Absorption curves of chlorophyll  $a$  from sunflower leaves and from diatoms by partition (●) and by adsorption (○). Solvent, methanol.

FIG. 4. Absorption curves of diatom extract and of chlorophyll  $a$ . Solvent, methanol.

FIG. 5. Absorption curves of extracts prepared from diatoms by various methods (see the text). Solvent, methanol.

FIG. 6. Absorption curves of extracts prepared from old and young cultures of diatoms. Solvent, methanol.

of hydrogen. A preparation of cells from another culture was extracted with acetone (80 per cent) and the resulting solution then evaporated at reduced pressure and room temperature. The residue obtained in this way was dissolved in methanol and its absorption curve was determined (Fig. 5).

The close agreement between all these absorption curves indicates that the deviation from the characteristic chlorophyll  $a$  curve is probably due to another pigment, or pigments, normally present in the living cells, rather than to a postmortem product. Furthermore, the spectral curve of methanol extracts of young, rapidly multiplying cultures of diatoms indicated a higher proportion of other pigment than that found in very old, more concentrated cultures (Fig. 6). The spectral absorption curves of methanol

extracts of brown algae (Fig. 7) were similar to those of *Nitzschia* extracts (Figs. 4 and 5). In Fig. 7, the absorption for *Fucus* indicates a smaller proportion of pigments other than chlorophyll *a*.

Fig. 8 shows a calculated curve representing the difference between the curve for diatom extract in Fig. 4 and that for chlorophyll *a*. This calculated curve is slightly influenced by yellow pigment (fucoxanthin) absorption below 610  $m\mu$  and is not continued below 570  $m\mu$  because of the increasing xanthophyll absorption. Indirect determination of green pigment absorption at shorter wave-lengths (24) was not feasible, because removal

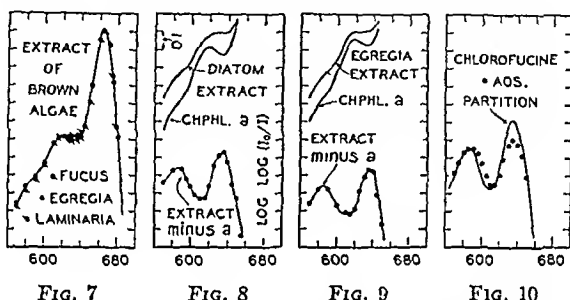


FIG. 7

FIG. 8

FIG. 9

FIG. 10

FIG. 7. Absorption curves of extracts of the brown algae, *Fucus furcatus*, *Egregia menziesii*, and *Laminaria andersonii*. Solvent, methanol.

FIG. 8. Calculated absorption curve representing the difference between absorption by a diatom extract and absorption by chlorophyll *a* in this extract. The lowest curve represents  $\log (\log (I_0/I))$  for extract minus  $\log (I_0/I)$  for chlorophyll *a* assuming from Fig. 4 that chlorophyll *a* absorbs 99 per cent of the light at 665  $m\mu$ .

FIG. 9. Calculated absorption curve representing the difference between absorption by an extract of *Egregia* and by chlorophyll *a* (cf. Fig. 8).

FIG. 10. Absorption curves of chlorofucine prepared by adsorption and by partition (see the text). Solvent, methanol.

of the chlorophylls through saponification resulted in alteration of several algal xanthophylls (7, 25). Fig. 9 shows a calculated curve for the brown alga *Egregia*. The calculated curves obviously do not correspond to chlorophyll *b*, but are in good agreement with the characteristic curve of chlorofucine prepared by partition of methanol extracts of *Nitzschia* (see below, Fig. 11). The agreement is particularly good in view of the fact that the calculation is based on relatively small differences between large absorption values (differences from one-twentieth to one-third of the total absorption of the extracts).

The results summarized in Figs. 4 to 9 lead to the conclusion that chlorofucine, rather than chlorophyll *b*, is the second green pigment of the diatoms and brown algae examined in this investigation.

*Absence of Chlorophyll b*—Chlorophyll *b*, in amounts up to 1 or 2 per cent of the amount of chlorophyll *a* (Fig. 3), would affect only slightly the spectral absorption of the extracted pigments and thus might escape detection in the original extract (Fig. 4). More sensitive tests for the presence of chlorophyll *b* were therefore employed.

Separate experiments revealed that extremely small quantities of chlorophyll *b* contained in extracts of barley leaves could be detected by adsorption upon columns of sugar (Sea Island). As little as 13  $\gamma$  of chlorophyll *b* formed a very distinct band on a column  $2 \times 12$  cm., 1.3  $\gamma$  formed a definite band on a column  $1.4 \times 12$  cm., and 0.03  $\gamma$  formed a barely perceptible band on a column  $0.2 \times 5$  cm. when petroleum ether with 1 per cent methanol was used to develop the chromatogram. Even when pure chlorophyll *a* was added in great excess to an extract of barley leaves containing 1.3  $\gamma$  of chlorophyll *b*, the latter formed a definite band in the presence of 2000 times this quantity of chlorophyll *a* (column  $1.4 \times 12$  cm.).

Pure chlorophyll *b* added to extracts of *Nitzschia* was readily recoverable by use of adsorption columns even when the amount of this pigment was less than 0.5 per cent of the amount of chlorophyll *a* present in the extracts. In this case a longer adsorption column ( $3 \times 20$  cm.) was necessary, because of the influence of other substances upon the adsorption. As a result of all these experiments, it is evident that chlorophyll *b* either is absent from diatoms and brown algae or is present only in traces.

When mixed with extracts of diatoms and adsorbed, chlorophyll *b* formed a green band below the principal orange band of fucoxanthin *a*. If the columns were then washed with petroleum ether containing 2 to 3 per cent of methanol, both the chlorophyll *b* and fucoxanthin were carried through the adsorbent, leaving chlorofucine behind as a diffuse green band. This comparative test indicates that the strongly adsorbed green pigment observed by Pace (16) under similar conditions was not chlorophyll *b* as he assumed. We believe that it, as well as a similar strongly adsorbed substance observed by Dutton and Manning (15) and occasionally by Montfort (14), was chlorofucine.

*Separation and Properties of Chlorofucine*—For determination of its spectral absorption properties, chlorofucine was prepared in the following way. Diatoms centrifuged from about 4 liters of culture suspension were extracted with about 80 ml. of absolute methanol containing 0.2 per cent dimethylaniline. The resultant pigment solution was diluted to 200 ml. with methanol, and, after the addition of 37 ml. of water, was extracted with 100 ml. of petroleum ether. As indicated already by Fig. 3, chlorophyll *a* was the only green constituent in the petroleum ether. The methanol layer containing the xanthophylls and chlorofucine was diluted with 160 ml. of water and extracted with 100 ml. of ether (U.S.P.) and then with five 75 ml.

portions of ether. The residual pale green methanol solution was treated with about 40 ml. of ether and a large excess of strong salt solution which caused the chlorofucine to dissolve in the ether. The chlorofucine, recovered by separation and evaporation of the ether, was dissolved in methanol, and its absorption was determined (Fig. 11).

It was difficult to obtain good preparations of chlorofucine from diatoms by this method. If extraction with ether was not carried on long enough, xanthophylls (principally fucoxanthins) contaminated the residual green pigment. If too many extractions were made, all the chlorofucine was re-

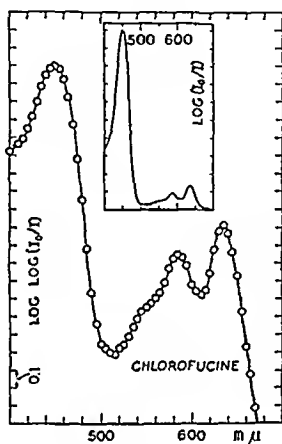


FIG. 11

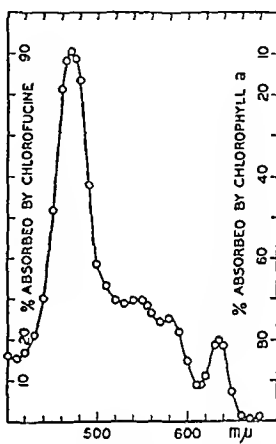


FIG. 12

FIG. 11. Absorption curves of chlorofucine prepared from diatoms by partition of the pigments between 50 per cent methanol and ether. The inset shows the same data plotted as  $\log(I_0/I)$ . Solvent, methanol.

FIG. 12. Relative proportions of light absorbed by chlorofucine and by chlorophyll *a* in a methanol extract of diatoms.

moved. With extracts of brown algae, fewer extractions with ether could be made before all the chlorofucine was removed from the methanol. However, with slight modifications of the method, satisfactory preparations of chlorofucine were also obtained from brown algae. Use of petroleum ether instead of ether for the partition often resulted in formation of emulsions that prevented separation of the two phases. These difficulties in the preparation of chlorofucine by partition probably account for some of the variable results obtained by earlier investigators (7-9).

Chlorofucine either from diatoms or from brown algae was easily prepared by adsorption on columns of sugar. The pigments were extracted

from the organisms with methanol and transferred to ether by partition. The ether solution was concentrated to a very small volume and diluted with petroleum ether. The resultant petroleum ether solution was passed through the adsorption column. (Direct transfer of chlorofucine from methanol to petroleum ether was incomplete, which may further explain some of the variable results obtained by others (8, 9, 13, 14).) When the chromatogram was developed with petroleum ether containing 2 to 4 per cent methanol, chlorofucine formed a pale green, diffuse band in the upper portion of the adsorbent. Below this, fucoxanthins formed orange bands. Other xanthophylls, chlorophyll  $a$ , and carotene passed rapidly through the column. Chlorofucine eluted from the green band with ether and transferred to methanol exhibited a spectral absorption curve that was significantly different from that of the same pigment prepared by partition (Fig. 10). Pigment obtained from the upper portion of the chlorofucine band on the column exhibited greater spectral variations than that obtained from the lowest portion. These differences were due, apparently, to alteration of the chlorofucine by adsorption upon the sugar rather than to the removal of other pigments. In one experiment, for example, chlorofucine prepared by partition was adsorbed on a column of sugar which was then washed with petroleum ether containing 2 per cent methanol and then with petroleum ether containing 4 per cent methanol. After 2.5 hours, all the pigments on the column were eluted with ether and alcohol, the ether was evaporated, and the spectral curve of the preparation was redetermined. It then showed changes corresponding to those observed with chlorofucine prepared directly by adsorption (Fig. 10).

Chlorofucine more like that prepared by partition was obtained when the pigments extracted from diatoms were adsorbed directly from petroleum ether containing 7.5 per cent  $n$ -propanol and 2 per cent dimethylaniline. Under these conditions traces of another green pigment similar to chlorofucine were observed above the chlorofucine band.

By means of adsorption, spectral absorption, or partition methods, chlorofucine was observed in all the brown algae that were examined; namely, *Fucus furcatus*, *Hesperophycus harveyanus*, *Pelvetiopsis limolata*, *Nereocystis pyrifera*, *Macrocystis integrifolia*, *Cystoseira osmundacca*, *Pterygophora californica*, *Laminaria andersonii*, *Egregia menziesii*. A more extensive investigation of *Laminaria* revealed chlorofucine both in old and young blades and in the stipes. When extracts of sunflower and of barley leaves were examined by means of the same methods employed with brown algae, chlorofucine was not observed. This result agrees with the early observations of Sorby (2).

Chlorofucine exhibits many properties that are similar to those of the common chlorophylls. It is altered by alcoholic KOH, yielding colored products insoluble in ether and petroleum ether and slightly soluble in

water. Chlorofucine obtained in solid form by evaporation of an ether solution is virtually insoluble in petroleum ether but readily soluble in methanol. It is insoluble in water and in dilute aqueous solutions of sodium carbonate and ammonia.

Solutions of chlorofucine are strongly fluorescent (11), although apparently less so than solutions of chlorophyll *a*. When the fluorescent light produced by exposure of an ether solution of the chlorofucine to light from a mercury arc was examined in the photoelectric spectrophotometer, a fluorescence band was observed at 635  $m\mu$ , near the absorption band in the red, but no fluorescence was observed at wave-lengths corresponding to the absorption band in the yellow. These results are in agreement with those of Wilschke (8) and of Dh  r   and Fontaine (11). There appeared to be a weak, secondary fluorescence maximum in ether at about 690  $m\mu$ .

Chlorofucine prepared by adsorption and dissolved in u.s.p. ether had absorption maxima at 627, 579.5, and 446  $m\mu$ , in satisfactory agreement with those observed spectroscopically by Tswett (6). For a similar preparation, the maxima in aqueous acetone (80 ml. of acetone to 20 ml. of water) were at 631, 581, and 446  $m\mu$ . As with chlorophylls *a* and *b*, the spectral absorption maxima of chlorofucine in both these solvents were more pronounced than those in alcohol solutions.

#### DISCUSSION

Because diatoms are the principal photosynthetic organisms over some four-fifths of the earth's surface, it appeared of interest to calculate the relative amounts of light absorbed by chlorofucine and chlorophyll *a* in methanol extracts of *Nitzschia closterium*. Results of these calculations are summarized in Fig. 12. It should be noted that light absorption by yellow pigments, which exceeds the absorption by chlorophyll in the blue-green region of the spectrum (15), does not enter into the calculation. Between 455 and 490  $m\mu$  the amount of light absorbed by chlorofucine was much greater than that absorbed by chlorophyll *a*. Similar results were obtained for extracts of several species of brown algae, another class that is widely distributed, and of great quantitative importance over large areas (*e.g.*, *Sargassum* in the Sargasso Sea). One is forced to the conclusion that chlorofucine may be an important pigment in the carbon economy of nature.

Demonstration of chlorofucine as a normal constituent of diatoms and brown algae indicates that pigments of the photosynthetic apparatus may be subject to greater variation than had been previously supposed. This leads to the supposition that further variations in the chemical nature of green pigments may be discovered in various plant species and mutants.

In general properties chlorofucine is so similar to chlorophylls *a* and *b* that it should probably be regarded as a chlorophyll type of pigment. Now that the existence of a so called chlorophyll *c* in higher plants has been

questioned (26) and the original claims of discovery have been retracted (18), it may become desirable to change the name chlorofucine to chlorophyll  $c$ , as first proposed by Wilschke (8).

#### SUMMARY

In addition to chlorophyll  $a$ , diatoms and brown algae contain chlorofucine, another green, chlorophyll-like pigment. Neither group of organisms contains detectable quantities of chlorophyll  $b$ . Chlorofucine is a normal constituent of the cells rather than a postmortem product. Although readily susceptible to chemical alteration, this green pigment has been separated from the other green and yellow pigments, and its spectral absorption properties have been determined. In certain regions of the spectrum, chlorofucine may absorb considerably more light than the chlorophyll  $a$  contained in the plant cells, an indication that this pigment may play an important rôle in the phenomenon of photosynthesis.

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# THE DISTRIBUTION OF THE BICARBONATE ION IN MAMMALIAN MUSCLE

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In the past 10 years the partition of electrolytes in mammalian muscle has been intensively investigated. As a result, it has been possible to reconcile the morphologically separate phases of muscle with its inorganic chemical constitution (1-6). This has led to the conclusions that the muscle cell contains a high concentration of potassium and phosphate, little, if any, sodium, and no chloride; while the fluid of the extracellular phase approximates in composition a serum ultrafiltrate and contains all of the muscle chloride and most of the sodium. More directly, Gersh (7) using histochemical methods and Dean (8) using ultramicroanalysis have provided direct evidence for the validity of these conclusions.

However, little attention has been paid to the bicarbonate ion in these studies, primarily because quantitative assessment of the bicarbonate distribution awaited solution of these problems. Fenn (9) and Stella (10) investigated the  $\text{CO}_2$  dissociation curve of frog muscle and Irving, Foster, and Ferguson (11) that of dog muscle. Shaw (12) and Brocklehurst and Henderson (13) have investigated the  $\text{CO}_2$ -combining properties of the entire body of the cat and man respectively. Meyerhof, Möhle, and Schulz (14) and Fenn and Mauer (15) have used the  $\text{CO}_2$  content of muscle to calculate the intracellular pH of frog muscle. In none of these studies has emphasis been placed upon the distribution of bicarbonate ion, nor were they combined with the inorganic analyses necessary for the evaluation of its distribution. Jacobs (16) studying certain plant cells and protozoa has demonstrated that the bicarbonate ion is effectively barred from passing the membranes of these cells. Gesell and Hertzman (17), working on the response of tissues to injected bicarbonate, arrived at an identical conclusion. On the other hand, Smith (18), using the effect of the bicarbonate ion and  $\text{CO}_2$  upon the amplitude of the beat of the excised turtle atrium, concluded that, in this tissue, the bicarbonate ion is freely permeable.

The low values found by investigators (9-11, 19, 20) for the total  $\text{CO}_2$  in muscles (7 to 12 mm per kilo) compared with the serum concentration of about 25 mm per kilo raise the question of whether muscle cells may not be impermeable to the bicarbonate ion as well as to the chloride ion. Since, in life, the muscle cell seems impermeable to anions (1), it would



seem paradoxical if the muscle cell were permeable to bicarbonate ion alone. The present work was therefore undertaken in an effort to determine the distribution of the bicarbonate ion between the muscle cell and its extracellular fluid and to study the effects of acidosis and alkalosis upon this distribution. The answer to this question is necessary for a description of the rôle played by tissues in regulation of the acid-base balance of the body. Furthermore, from the data obtained in these experiments, it has been possible to estimate the intracellular pH of cat muscle, both under normal conditions and under conditions of disturbed acid-base balance.

### *Experimental Procedure*

Cats, anesthetized with 0.05 gm. of dial per kilo, were used as experimental animals. Approximately 1 hour was allowed for stabilization of the anesthesia and respiration before a sample of blood was taken from the femoral vein and a sample of muscle from the quadriceps femoris of the same side. Acidosis and alkalosis were then induced by the intravenous injection of acid and alkaline isotonic salt solutions (the composition is given in Tables II and III) into the femoral vein at the rate of 5 to 8 cc. per minute. At the end of the injection, the animals were left covered and warm on the table for 1 hour to insure equilibration of the injected solution. At the end of this time, a second sample of blood and muscle was taken from the opposite leg. A series of animals was also rendered alkalotic by the intraperitoneal injection of sodium bicarbonate solution according to the procedure of Yannet (21). In this series of experiments, it was found that 6 hours of equilibration were necessary to obtain the desired state of alkalosis. Because of the difficulty encountered in obtaining stable anesthesia during this length of time, it became necessary to obtain the control blood and muscle almost immediately after induction of anesthesia rather than to wait for an hour as was done in the intravenous experiments. As a result, the control period determinations on the intraperitoneally injected animals are not strictly comparable to those obtained on the intravenous series.

### *Chemical Methods*

Whole blood  $\text{CO}_2$ , serum pH, and percentage of red cells were determined in triplicate by the micromethod of Shock and Hastings (22). Serum pH as determined by this method on cat serum was compared with glass electrode determinations on the same serum in a number of instances. The values agreed to 0.03 pH.

The water content of the serum was determined by drying 0.5 gm. samples overnight in an oven at  $100^\circ$ . Chlorides were determined by the method of Wilson and Ball (23) on the resulting serum solids.

Weighed amounts of muscle (4 to 5 gm.) were finely minced in 25 cc. digestion flasks and the water content determined by drying to constant weight at 100°. The fat was extracted from the dried tissue by the method of Hastings and Eichelberger (3) and, after the defatted tissue was weighed, it was returned to the flask. Silver nitrate was then added to the dried material and allowed to soak into the tissue overnight before nitric acid digestion was carried out. Titration with potassium thiocyanate was carried out in duplicate on 5 cc. aliquots of the nitric acid digest.

The amount of blood in the tissues was determined by extracting with 0.4 per cent ammonia and determining the hemoglobin content of the extract colorimetrically (3).

Total tissue  $\text{CO}_2$  was determined by the method of Danielson and Hastings (20). With practice, it was found that a piece of muscle could be transferred from the analysis tube in 40 seconds, thus minimizing the loss of  $\text{CO}_2$ . Analyses were made in duplicate. The average difference between duplicates was 0.4 mm per kilo. Standard sodium carbonate solutions analyzed by this method gave results within 0.5 per cent of that obtained by the procedure of Van Slyke and Neill (24). The larger error in the muscle analyses is attributed to the difficulty of sampling equal portions of tissue. In order to determine the over-all blank for the method as applied to muscle, pieces of muscle were equilibrated with several changes of  $\text{CO}_2$ -free salt solution at 6° for 3 to 4 hours and analyzed by this method. The  $\text{CO}_2$  content of muscle equilibrated in this manner amounted to only 0.05 mm per kilo, showing that the method was specifically valid for  $\text{CO}_2$  determination in this tissue.

### *Calculation*

Whole blood  $\text{CO}_2$  values were converted to their corresponding serum values by means of the nomogram of Van Slyke and Sendroy (25) with the hematocrit values found and assuming 50 per cent oxygen saturation of the venous blood. Serum  $\text{CO}_2$  tension and  $[\text{HCO}_3^-]$  were calculated according to the Henderson-Hasselbalch equation. The extracellular space of the muscle was calculated according to the method of Hastings and Eichelberger (3) from the water and chloride data. With the value so obtained, the  $\text{CO}_2$  content of this phase was calculated, assuming a distribution ratio between serum and extracellular fluid of 0.95 (26). The  $\text{H}_2\text{CO}_3$  of the extracellular and intracellular phase was calculated, assuming equality of the  $\text{CO}_2$  tension in these phases with the venous serum. While a gradient of tension must theoretically exist between the extra- and intracellular phases and venous serum; the high value for the diffusion coefficient of  $\text{CO}_2$  and the proximity of the capillary wall (27) make it unlikely that the gradient between the serum and the tissue is significant in magnitude. The total  $\text{CO}_2$  content of the whole muscle minus the

calculated  $\text{CO}_2$  content of the extracellular space is taken as the  $\text{CO}_2$  content of the cells. The cell bicarbonate is calculated as total  $\text{CO}_2$  minus  $\text{H}_2\text{CO}_3$ . (This value would include any  $\text{CO}_2$  present as carbamate.) From these derived values, the intracellular pH may be calculated by the Henderson-Hasselbalch equation.

The value for the solubility coefficient of  $\text{CO}_2$  in serum used was 0.553 cc. per gm. of serum water (28). The coefficient for the water of the extracellular space used was 0.540 cc. per gm. of extracellular water (28). For the water of the intracellular space, the value of 0.592 cc. per gm. of intracellular water was employed (29).

The value for  $\text{pK}'$  of serum of 6.10 has been used (30). For the muscle cell, the value of  $\text{pK}'$  of 6.10 as determined by Danielson, Chu, and Hastings (29) has been taken.

The following representative calculation will illustrate the method of calculation of the intracellular bicarbonate and pH from the experimental data. In this calculation, parentheses refer to concentration per kilo of serum, extracellular or intracellular phase, and whole tissue, brackets to concentrations per kilo of water of the respective phases or whole tissue. The subscripts,  $S$ ,  $E$ ,  $C$ , refer to serum, extracellular, and intracellular phases, respectively, while  $T$  refers to the tissue as a whole. The subscript,  $TE$ , refers to the amount of a constituent present in the extracellular phase per kilo of whole tissue. The subscript,  $TC$ , has a comparable meaning with respect to constituents of the intracellular phase.

### Experimental Data

$(\text{H}_2\text{O})_S$	= 925.0 gm. per kilo
$(\text{H}_2\text{O})_T$	= 770.0 " " "
$(\text{Cl})_S$	= 112.5 m.eq. per kilo
$(\text{Cl})_T$	= 15.7 " " "
$(\text{CO}_2)_S$	= 25.6 mm per kilo
$(\text{CO}_2)_T$	= 12.6 " " "
$\text{pH}_S$ at $38^\circ$	= 7.30
$(\text{HCO}_3)_S$	= 24.0 mm per kilo
$\text{pCO}_2$	= 51.7 mm. of Hg

The extracellular phase ( $E$ ) is calculated as follows:

$$[\text{Cl}]_S = \frac{(\text{Cl})_S \times 1000}{(\text{H}_2\text{O})_S} = \frac{112.5 \times 1000}{925.0} = 121.6 \text{ m.eq. Cl per kilo } \text{H}_2\text{O}_S$$

$$[\text{Cl}]_E = \frac{[\text{Cl}]_S}{0.95} = \frac{121.6}{0.95} = 128.0 \text{ m.eq. Cl per kilo } \text{H}_2\text{O}_E$$

$$(\text{H}_2\text{O})_{TE} = \frac{(\text{Cl})_T \times 1000}{[\text{Cl}]_E} = \frac{15.7 \times 1000}{128.0} = 122.6 \text{ gm. } \text{H}_2\text{O}_E \text{ per kilo } T$$

$$E = \frac{(\text{H}_2\text{O})_{TE}}{0.99} = \frac{122.6}{0.99} = 123.8 \text{ gm. } E \text{ per kilo } T$$

The value 0.99 is taken as the water concentration in the extracellular phase in gm. of  $H_2O$  per kilo of this phase.

The water of the intracellular phase is calculated from the determined total muscle water and the calculated extracellular water as follows:

$$(H_2O)_{TC} = (H_2O)_T - (H_2O)_{TE} = 770.0 - 122.6 = 647.4 \text{ gm. } H_2O \text{ per kilo } T$$

The water concentration, per kilo of cells, is

$$(H_2O)_C = \frac{(H_2O)_{TC} \times 1000}{1000 - E} = \frac{647.4 \times 1000}{876.2} \approx 739 \text{ gm. } H_2O \text{ per kilo } C$$

The concentrations of  $[H_2CO_3]_E$  and  $[HCO_3]_E$  in the extracellular phase are calculated with a distribution ratio for the bicarbonate ion of 0.95 and assuming equality of  $CO_2$  tension in the serum and extracellular phase.

$$\begin{aligned} [H_2CO_3]_E &= \frac{pCO_2}{760} \times \frac{1000}{22.26} \times \alpha^b_{CO_2, E} \\ &= \frac{51.7}{760} \times \frac{1000}{22.26} \times 0.540 \\ &= 1.65 \text{ mM } H_2CO_3 \text{ per kilo } H_2O_E \end{aligned}$$

$$[HCO_3]_E = \frac{(HCO_3)_S \times 1000}{(H_2O)_S} = \frac{24.0 \times 1000}{925.0} = 26.0 \text{ m.eq. } HCO_3 \text{ per kilo } H_2O_S$$

$$[HCO_3]_E = \frac{[HCO_3]_S}{0.95} = \frac{26.0}{0.95} = 27.4 \text{ m.eq. } HCO_3 \text{ per kilo } H_2O_E$$

The concentrations of  $H_2CO_3$  and  $HCO_3$  in the extracellular phase being known, the amount of  $CO_2$  in this phase may be calculated

$$(H_2CO_3)_{TE} = \frac{[H_2CO_3]_E \times (H_2O)_{TE}}{1000} = \frac{1.65 \times 122.6}{1000} = 0.20 \text{ mM } H_2CO_3 \text{ in } E \text{ per kilo } T$$

$$(HCO_3)_{TE} = \frac{[HCO_3]_E \times (H_2O)_{TE}}{1000} = \frac{27.4 \times 122.6}{1000} = 3.36 \text{ m.eq. } HCO_3 \text{ in } E \text{ per kilo } T$$

$$(CO_2)_{TE} = (H_2CO_3)_{TE} + (HCO_3)_{TE} = 0.20 + 3.36 = 3.56 \text{ mM } CO_2 \text{ in } E \text{ per kilo } T$$

The  $CO_2$  assignable to the intracellular phase is thus

$$(CO_2)_{TC} = (CO_2)_T - (CO_2)_{TE} = 12.6 - 3.56 = 9.04 \text{ mM } CO_2 \text{ in } C \text{ per kilo } T$$

The  $CO_2$  of the cells per kilo of water of the cells is calculated

$$[CO_2]_C = \frac{(CO_2)_{TC} \times 1000}{(H_2O)_{TC}} = \frac{9.04 \times 1000}{647.4} = 14.0 \text{ mM } CO_2 \text{ per kilo } H_2O_C$$

Again, assuming equality of  $pCO_2$  inside and outside the cell, the intracellular concentrations of  $[H_2CO_3]_C$  and  $[HCO_3]_C$  are calculated

$$\begin{aligned}
 [\text{H}_2\text{CO}_3]_c &= \frac{p\text{CO}_2}{760} \times \frac{1000}{22.26} \times \alpha^0 \text{CO}_2^c \\
 &= \frac{51.7}{760} \times \frac{1000}{22.26} \times 0.592 = 1.81 \text{ mm H}_2\text{CO}_3 \text{ per kilo H}_2\text{O}_c
 \end{aligned}$$

$$[\text{HCO}_3]_c = [\text{CO}_2]_c - [\text{H}_2\text{CO}_3]_c = 14.0 - 1.81 = 12.2 \text{ mm HCO}_3 \text{ per kilo H}_2\text{O}_c$$

The distribution ratio ( $r$ ) of the  $\text{HCO}_3$  ion between extracellular and intracellular phase is

$$r = \frac{[\text{HCO}_3]_c}{[\text{HCO}_3]_E} = \frac{12.2}{27.4} = 0.45$$

The intracellular pH is calculated from the Henderson-Hasselbalch equation with the value for the  $\text{pK}'$  of the muscle fiber of 6.10

$$\begin{aligned}
 \text{pH}_c &= 6.10 + \log \frac{[\text{HCO}_3]_c}{[\text{H}_2\text{CO}_3]_c} \\
 &= 6.10 + \log \frac{12.20}{1.81} \\
 \text{pH}_c &= 6.93
 \end{aligned}$$

### Results

*Control Experiments*—In Table I are shown the analytical and derived data from two cats carried through the experimental procedure but without the injection of any fluid, and of one animal injected with 150 cc. per kilo of a solution containing 21 mm per liter of  $\text{NaHCO}_3$  and 134 mm per liter of  $\text{NaCl}$ , adjusted to approximately pH 7.4 with  $\text{CO}_2$ . These experiments show the variations due to fluctuations in the state of the anesthesia, the general state of the animal, and that due to the problem of the sampling of tissues over the experimental period. Such changes are small; the cell  $\text{HCO}_3^-$  remains as constant as can be expected with the analytical method used. The only significant change in the injected animal (No. 21) is the increase in the extracellular space ( $E$ ) without significant alteration in its intracellular water ( $\text{H}_2\text{O}$ ) $_c$ , intracellular  $\text{HCO}_3$ , and intracellular pH.

From our data on normal skeletal muscle of anesthetized cats, the mean values for the intracellular pH and for  $[\text{HCO}_3^-]_c/[\text{HCO}_3^-]_E$  are 6.93 and 0.53 respectively.

Similar data on normal skeletal muscle of anesthetized rabbits previously obtained by Dr. I. S. Danielson and Dr. H. I. Chu, of this laboratory, yield values for intracellular pH of 6.70 and for  $[\text{HCO}_3^-]_c/[\text{HCO}_3^-]_E$  of 0.30.

*Acidosis Experiments*—In Table II are shown the analytical and derived

data obtained from four cats injected with a solution containing 10 mm per liter of HCl and 154 mm per liter of NaCl. Intense metabolic acidosis was produced, the pH and  $\text{HCO}_3^-$  of the serum falling, accompanied by a corresponding rise in the serum chloride. The noteworthy changes in the muscle are a marked increase in the extracellular space. No significant change in the water concentration of the intracellular space occurs and only

TABLE I  
*Control Experiments*

Analyses of serum and muscle of control cats. The animals of Experiments 1 and 7 were uninjected; that of Experiment 21 was injected with 100 cc per kilo of a solution containing 135 mm per liter of NaCl and 21 mm per liter of  $\text{NaHCO}_3$ , adjusted to pH 7.40 with carbon dioxide. 90 minutes were allowed to elapse between taking samples A and B in the uninjected animals, and 60 minutes in the injected animals after the end of the injection before sample B was taken. Column A refers to the control period, column B to the experimental period.

Experimental Data													
Experiment No	Tissue	H <sub>2</sub> O, gm. per kilo		pH		CO <sub>2</sub> , mm per kilo		pCO <sub>2</sub> , mm		[HCO <sub>3</sub> ], m eq per kilo H <sub>2</sub> O		[Cl], m eq per kilo H <sub>2</sub> O	
		A	B	A	B	A	B	A	B	A	B	A	B
1	Serum	910	899	7.24	7.24	22.8	23.2	52.6	53.5	23.4	24.1	103.1	101.1
	Muscle	738	746			12.2	11.4					10.4	11.9
7	Serum	915	914	7.24	7.23	20.9	19.0	48.7	44.2	21.3	19.4	118.4	116.0
	Muscle	765	767			10.5	10.1					12.5	11.7
21	Serum	929	952	7.30	7.30	18.7	18.9	38.0	38.2	19.0	18.7	115.5	119.2
	Muscle	762	778			10.2	10.2					12.6	17.0
Derived Data													
	E, gm. per kilo muscle		H <sub>2</sub> O, gm. per kilo cells		[HCO <sub>3</sub> ] <sub>c</sub> , m eq per kilo H <sub>2</sub> O cells		pH, cells		[HCO <sub>3</sub> ] <sub>c</sub> / [HCO <sub>3</sub> ] <sub>E</sub>				
	A	B	A	B	A	B	A	B	A	B			
1	87.9	101.0	715	719	13.3	11.6	6.96	6.88	0.54	0.46			
7	92.5	88.3	741	744	10.8	10.6	6.90	6.94	0.48	0.52			
21	96.9	130.1	739	746	11.0	10.3	7.02	6.99	0.55	0.52			

a slight decrease in the total  $\text{CO}_2$  and intracellular  $\text{HCO}_3^-$ . The distribution ratio of the bicarbonate ion between the extracellular fluid and the intracellular fluid increases greatly owing to the pronounced decrease produced in the extracellular fluid  $\text{HCO}_3^-$  concentration. No significant change occurs in the intracellular pH.

*Alkalosis Experiments*—In Table III are shown the results obtained after the intravenous injection of a solution containing 40 mm per liter of

$\text{NaHCO}_3$  and 114 mm per liter of  $\text{NaCl}$ . A definite series of events was observed in a large series of animals so treated. Without exception, respiration was greatly increased both in rate and in volume. Toward the end of the injection period, or very shortly following the cessation of the injection, marked tetany appeared and persisted for 1 to 2 hours. As was to be expected, the serum pH increased, but contrary to expectation, the

TABLE II

Analyses of serum and muscle of animals injected intravenously with 100 cc per kilo of body weight of a solution containing 10 mm per liter of  $\text{HCl}$  and 154 mm per liter of  $\text{NaCl}$  60 minutes were allowed to elapse between the end of the injection and securing sample B. Column A refers to the control period, column B to the experimental period.

*Experimental Data*

Experiment No	Tissue	$\text{H}_2\text{O}$ , gm per kilo		pH		$\text{CO}_2$ , mm per kilo		$p\text{CO}_2$ , mm		$[\text{HCO}_3^-]$ , m eq per kilo $\text{H}_2\text{O}$		$[\text{Cl}^-]$ m eq per kilo $\text{H}_2\text{O}$	
		A	B	A	B	A	B	A	B	A	B	A	B
8	Serum	921	945	7 27	7 13	21 8	13 7	47 1	39 9	22 2	13 3	117 0	129 1
	Muscle	765	779			12 5	10 5					11 7	17 0
17	Serum	923	946	7 18	7 00	19 5	12 5	50 9	47 6	19 0	11 8	114 9	124 3
	Muscle	767	780			11 4	9 8					16 8	20 6
18	Serum	911	942	7 30	7 20	22 9	13 8	46 4	34 7	23 6	13 6	112 1	125 2
	Muscle	757	775			10 9	8 78					16 3	22 7
19	Serum	921	946	7 25	7 07	20 6	14 1	46 5	46 2	20 9	13 5	117.0	127 5
	Muscle	767	783			11 6	9 5					14 1	22 6

*Derived Data*

	$E$ , gm per kilo muscle		$\text{H}_2\text{O}$ , gm per kilo cells		$[\text{HCO}_3^-]_c$ , m eq per kilo $\text{H}_2\text{O}$ cells		pH, cells		$\frac{[\text{HCO}_3^-]_c}{[\text{HCO}_3^-]_s}$	
	A	B	A	B	A	B	A	B	A	B
8	88 4	119 2	743	750	13 6	11 8	7 02	7 03	0 59	0 84
17	129 1	150 0	734	741	11 9	10 6	6 92	6 90	0 59	0 85
18	127 0	164 0	724	732	10 5	9 0	6 91	6 97	0 42	0 63
19	117 0	127 5	740	740	12 2	9 6	6 97	6 87	0 55	0 68

serum bicarbonate was only slightly elevated and in several animals had actually decreased at the end of the equilibration period of 1 hour. The serum chloride level remained essentially unchanged. Because of the increase in the blood lactic acid known to occur in conditions of alkalosis, several determinations of the blood lactic acid during the control period and at the time of taking the second sample of blood were made. No increases in the blood lactic acid were found in our experiments. In all cases, the total muscle  $\text{CO}_2$  is lower after the injection than before, as is

the calculated cell bicarbonate. The concentration of water in the intracellular phase is increased significantly in some of the experiments. The shifts of water in the muscle in these experiments are the same as those previously described by Hastings and Eichelberger (3). The distribution ratio of the  $\text{HCO}_3^-$  decreased, but the intracellular pH did not change significantly.

Animals subjected to the intraperitoneal injection of bicarbonate solutions presented an entirely different picture. The data so obtained are

TABLE III

Analyses of serum and muscle of animals injected intravenously with 100 cc. per kilo of a solution containing 40 mm per liter of  $\text{NaHCO}_3$  and 114 mm per liter of  $\text{NaCl}$ . 60 minutes were allowed to elapse between the end of the injection and the time of securing sample B.

Experimental Data													
Experiment No.	Tissue	H <sub>2</sub> O, gm. per kilo		pH		CO <sub>2</sub> , mm per kilo		pCO <sub>2</sub> , mm.		[HCO <sub>3</sub> ], m eq per kilo H <sub>2</sub> O		[Cl], m eq per kilo H <sub>2</sub> O	
		A	B	A	B	A	B	A	B	A	B	A	B
12	Serum	925	946	7.30	7.45	25.6	25.8	51.7	37.4	26.0	26.1	112.5	116.0
	Muscle	770	782			12.6	9.8					15.7	17.9
3	Serum	919	945	7.29	7.39	22.4	23.1	46.5	38.3	22.9	23.2	114.0	113.0
	Muscle	751	772			12.9	11.6					13.2	15.5
6	Serum	916	934	7.20	7.38	20.3	20.9	50.7	36.6	20.6	20.3	117.4	117.5
	Muscle	763	778			10.7	9.8					10.5	16.1

Derived Data										
	E, gm. per kilo muscle		H <sub>2</sub> O, gm. per kilo cells		[HCO <sub>3</sub> ] <sub>c</sub> , m eq per kilo H <sub>2</sub> O cells		pH, cells		$\frac{[\text{HCO}_3]_c}{[\text{HCO}_3]_E}$	
	A	B	A	B	A	B	A	B	A	B
12	123.5	140.0	737	748	12.1	7.7	6.93	6.81	0.44	0.28
3	102.0	124.1	724	740	14.3	11.7	7.04	7.04	0.59	0.48
6	78.7	123.0	744	748	11.1	9.7	6.90	6.97	0.52	0.45

presented in Table IV. Little or no visible stimulation of respiration occurred and only one animal showed any evidence of tetany. Typical metabolic alkalosis of a marked grade was present. The serum  $\text{HCO}_3^-$  concentration was approximately doubled, with a corresponding decrease in the serum chloride level. The pH and the  $\text{CO}_2$  tension of the serum were increased. In all experiments, there was an increase in the total muscle  $\text{CO}_2$ , although the bicarbonate concentration of the cells on a water basis,  $[\text{HCO}_3^-]_c$ , decreased in the experiments with isotonic  $\text{NaHCO}_3$  (Experiments 23, 25, 26). The bicarbonate distribution ratio between extra-



cellular fluid and cells decreased greatly. A considerable drop in the pH of the intracellular phase occurred. In Experiment 22, the bicarbonate solution injected contained glucose in a concentration of 25 per cent. In this experiment, the extracellular fluid, *E*, increased greatly and the intracellular fluid decreased. This is in accord with the findings of previous investigators (31, 32). In the remaining experiments of this series in

TABLE IV

Analyses of serum and muscle of animals injected intraperitoneally with 100 cc per kilo of a solution containing 150 mM per liter of  $\text{NaHCO}_3$ . Blood and muscle samples B were taken after 6 hours of equilibration. In Experiment 22, the bicarbonate solution was made hypertonic by the addition of 250 gm per liter of glucose

*Experimental Data*

Experiment No	Tissue	$\text{H}_2\text{O}$ , gm per kilo		pH		$\text{CO}_2$ , mM per kilo		$p\text{CO}_2$ , mm.		$[\text{HCO}_3^-]$ , m eq per kilo $\text{H}_2\text{O}$		$[\text{Cl}^-]$ , m eq per kilo $\text{H}_2\text{O}$	
		A	B	A	B	A	B	A	B	A	B	A	B
22	Serum	915	914	7.25	7.50	22.5	46.0	50.6	60.0	23.0	48.1	114.0	83.3
	Muscle	766	753			10.0	15.1					16.9	15.4
23	Serum	920	923	7.35	7.55	19.7	43.4	35.6	50.7	20.3	45.4	115.5	91.0
	Muscle	775	784			9.1	11.7					18.1	13.0
25	Serum	917	916	7.35	7.50	22.0	42.6	38.9	54.3	22.0	44.9	114.1	90.7
	Muscle	762	771			9.4	11.5					17.3	14.6
26	Serum	926	930	7.35	7.50	19.3	38.4	35.0	50.0	19.7	39.8	112.6	89.9
	Muscle	778	779			10.1	13.2					22.8	17.8

*Derived Data*

	<i>E</i> , gm per kilo muscle		$\text{H}_2\text{O}$ , gm per kilo cells		$[\text{HCO}_3^-]_c$ , m eq per kilo $\text{H}_2\text{O}$ cells		pH, cells		$\frac{[\text{HCO}_3^-]_c}{[\text{HCO}_3^-]_E}$	
	A	B	A	B	A	B	A	B	A	B
22	130	162	731	705	8.7	9.2	6.80	6.74	0.36	0.18
23	138	126	740	753	8.3	6.8	6.92	6.78	0.37	0.14
25	134	141	725	735	8.5	5.5	6.90	6.56	0.37	0.12
26	180	177	730	731	9.1	7.6	6.97	6.74	0.44	0.18

which an isotonic bicarbonate solution was employed, little change occurred in the proportion of extracellular phase.

As noted under the section on experimental procedure, the control analyses on the intraperitoneally injected animals were carried out on tissues taken at an earlier period in the experiment than in the intravenous experiments. The calculated values for the intracellular  $\text{HCO}_3^-$  are somewhat lower in these experiments than in the intravenous ones. This may be related to the lower  $\text{CO}_2$  tensions of the blood in the experiments of Table IV.

## DISCUSSION

The experiments just presented had for their purpose the determination of the intracellular  $\text{HCO}_3$  concentration and pH when the acid-base balance of the circulating blood plasma, and presumably the environmental fluid of the cells, had been altered. The changes produced in the acid-base balance of the plasma included both metabolic acidosis and metabolic alkalosis. A mild alkalosis was produced by the intravenous injection of  $\text{NaHCO}_3$ , and a severe alkalosis by the intraperitoneal injection of  $\text{NaHCO}_3$ .

The small influence of varying the plasma  $\text{HCO}_3$  concentration on the intracellular  $\text{HCO}_3$  is shown graphically in Fig. 1. The height of each

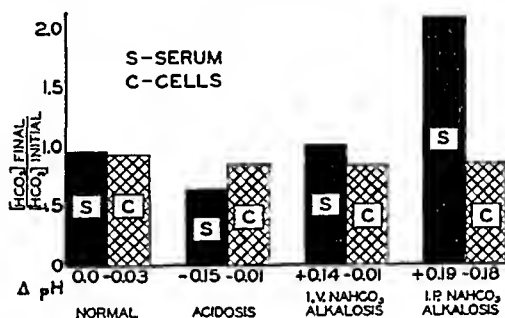


Fig. 1. Serum bicarbonate and intracellular bicarbonate in acidosis and alkalosis. The height of each column represents the ratio of the final  $\text{HCO}_3$  concentration to the initial concentration. The left-hand column of each pair represents the ratio for serum and the right-hand the ratio for the intracellular phase of muscle.  $\Delta \text{pH}$  denotes the change in pH of the respective phases from the initial to final condition. I. P. designates intraperitoneal and I. V., intravenous administration.

column represents the ratio of the final  $[\text{HCO}_3]$  concentration to the initial  $[\text{HCO}_3]$ . The ratios have been calculated from the mean  $\text{HCO}_3$  values for each series of experiments. The left-hand column of each pair denotes the  $[\text{HCO}_3]$  ratio for serum, the right-hand column the corresponding ratio for the intracellular phase of the muscle.

It is seen that, although the serum  $\text{HCO}_3$  was reduced in the acidosis experiments to 60 per cent of its initial value and increased in the alkalosis experiments produced by intraperitoneal injection to 100 per cent above its initial value, the ratios of the final intracellular  $\text{HCO}_3$  concentration to the initial  $\text{HCO}_3$  remained remarkably constant. There was in all instances a small average decrease in intracellular  $\text{HCO}_3$ , its final value being 85 per cent of the initial value in the three types of experiments in which the acid-base balance was displaced and 90 per cent in the control experiments.

*These data are therefore interpreted as indicating that the intracellular  $\text{HCO}_3$  concentration of muscle is not directly influenced by the concentration of  $\text{HCO}_3$  in the extracellular fluid.*

If the above conclusion, that the intracellular  $\text{HCO}_3$  remains essentially constant, is valid, it follows that the intracellular pH is influenced largely by changes in  $\text{CO}_2$  tension. Although no direct determinations of intracellular  $\text{CO}_2$  tension in tissues are yet practicable, it is possible to calculate the maximum intracellular pH assuming the intracellular  $\text{CO}_2$  tension is equal to that of venous blood. This has been done in the experiments just presented and the differences between the means of the initial and final pH values for the serum and for the intracellular phase have been listed at the bottom of Fig. 1. When the difference in  $\text{CO}_2$  tensions was slight and in the same direction as the change in intracellular  $\text{HCO}_3$ , as in the case of the intravenous injection of acid and alkaline solutions, the resulting change in intracellular pH was negligible. When the change in  $\text{CO}_2$  tension of the blood was great and in the opposite direction to that of the intracellular  $\text{HCO}_3$ , as happened after the intraperitoneal injection of  $\text{NaHCO}_3$ , the change in intracellular pH was large. Indeed, although the serum pH increased 0.19 in this latter series of experiments, the intracellular pH decreased by 0.18.

It would thus appear that the intracellular pH of muscle is influenced primarily by the  $\text{CO}_2$  tension of the environmental fluid of the cells and not primarily by its pH. It may therefore be concluded that a condition of alkalosis accompanied by an increased  $\text{CO}_2$  tension may be characterized by an intracellular acidosis. Whether or not a condition of acidosis associated with a decreased  $\text{CO}_2$  tension would be accompanied by an intracellular alkalosis awaits investigation.

#### SUMMARY

Experiments on anesthetized cats are described in which the acid-base balance of the animals was varied by the intravenous and intraperitoneal injection of acid and alkaline solutions and the changes in the blood and muscle  $\text{CO}_2$ , water, and chloride studied.

A method is described whereby these data may be used to calculate the intracellular bicarbonate and pH of the muscle cell in the anesthetized animal.

It was shown that the intracellular bicarbonate remains relatively unchanged despite wide changes in the extracellular bicarbonate and it is concluded that the muscle cell is normally impermeable to the bicarbonate ion. The effect of such an impermeability upon the intracellular pH is discussed.

The mean intracellular pH of the skeletal muscle of the cat is calculated to be  $\text{pH } 6.93 \pm 0.12$ .

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# AN IN VITRO STUDY OF CARBON DIOXIDE EQUILIBRIA IN MAMMALIAN MUSCLE

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In the preceding communication, which dealt with the carbon dioxide content of mammalian muscle, data were presented which could best be explained by assuming that the muscle fiber is impermeable to bicarbonate ion. These experiments were performed on cats rendered acidotic and alkalotic by the injection of acid and alkaline solutions. In such experiments with intact animals, it was difficult to control the condition of the serum with any precision because of strong compensatory physiological reactions to the acid or basic shifts. Nor was it found possible to change the reaction of the serum, and hence of the fluid around the fibers, over a wide range without causing the death of the animal.

It was evident that by producing greater changes in the bicarbonate ion and carbon dioxide tension of the fluid bathing the muscle fibers, more definite evidence would be obtained as to the relationships existing between the intra- and extracellular hydrogen and bicarbonate ion concentrations. By equilibrating skeletal muscle with various solutions *in vitro*, it was possible (a) to establish better control over the fluid environment of the skeletal muscle fibers studied, and (b) to vary the composition of this environment over wide limits.

The results of these *in vitro* experiments appear to confirm the *in vivo* studies.

## *Experimental Procedure and Methods*

One-half of the entire abdominal musculature of the rat was used for each equilibration. The full thickness of the musculature was removed with care to avoid trauma. Wherever possible, incisions were made through raphe or tendons to avoid cutting across fibers. In this manner, two sheets of muscle each roughly  $40 \times 50 \times 2$  mm. were obtained from each rat.

These muscle preparations were equilibrated in 125 cc. Erlenmeyer flasks completely filled with various salt solutions. The equilibrations were carried out anaerobically by saturating the solutions with nitrogen and subsequently admitting  $\text{CO}_2$  until the desired pH was attained. The flasks were slowly rotated either for 45 minutes at  $36^\circ$ , or for 2.5 hours at  $6^\circ$ . From preliminary experiments, it had been found that these times

permitted equilibrium to be established without drastic alterations in the permeability characteristics of the muscle cells. All the solutions used contained cations in the following concentrations, in milliequivalents per liter, Na 140, K 4, Ca 1.5, Mg 1.5.

The corresponding anions of the solution were  $\text{HCO}_3$  or Cl. The pH was varied by varying the  $\text{HCO}_3$  concentration or  $\text{CO}_2$  tension. In order to prevent the pH from varying during the course of an experiment, owing to acid formation, all solutions contained the following buffer mixture, glycylglycine 4 mM per liter, phosphate 1 mM per liter, and acetate 5 mM per liter.

After the termination of the equilibrations, two muscle samples were removed in each case, one for the measurement of the total  $\text{CO}_2$ , and the other for the determination of the water, fat, and chloride concentrations. These two samples were taken from the obliquus externus, obliquus internus, and transversus in a region where only a small proportion of the muscle fibers had been severed initially. The excess equilibration fluid was rapidly removed from the samples with filter paper. The samples used for  $\text{CO}_2$  analysis were handled as rapidly as possible in order to minimize loss of  $\text{CO}_2$ .

In spite of the period of anaerobiosis, these preparations were, in many respects, still excitable at the end of the equilibrations. For example, the muscle fibers were apparently still free of chloride ion as in the living animal. Occasionally, the muscles still responded to mechanical stimulation when removed from the equilibration medium.

In order to obtain truly dead muscle, as a control, preparations were heated for 60 minutes at 50–54°. These samples were then equilibrated with various solutions as in the case of the original, fresh muscle.

The pH values of the equilibration media were determined with the glass electrode at 25°. From these values, the pH values at the respective equilibration temperatures were calculated. The other chemical methods and methods of calculation have been described in the preceding communication. In calculating the amount of extracellular fluid, the equilibration medium and the extracellular fluid have been regarded as identical.

### Results

In Table I are muscle data obtained following 2.5 hours equilibration at 6° with solutions of not widely different  $\text{CO}_2$  tensions, but which contained from 0 to 87.0 mM of bicarbonate ion per liter.

The bicarbonate ion concentration in the fibers completely failed to respond to the wide differences in bicarbonate concentration of the external solutions. There was actually less bicarbonate observed in the fibers

TABLE I

*Muscle Data Obtained Following 2.5 Hours Equilibration at 6° with Solutions of Nearly Constant CO<sub>2</sub> Tension, but with Varying Bicarbonate Concentration*

<i>Experimental Data</i>			
	pH	CO <sub>2</sub>	(H <sub>2</sub> O) <sub>T</sub>
		<i>mm per kg.</i>	<i>gm. per kg.</i>
Solution.....	8.03	88.5	
Tissue.....		20.4	797
Solution.....	7.78	72.4	
Tissue.....		15.9	777
Solution.....	7.57	40.4	
Tissue.....		7.1	777
Solution.....	7.43	21.0	
Tissue.....		9.8	803
Solution.....	6.64	4.7	
Tissue.....		5.8	799
Solution.....	5.59	1.8	
Tissue.....		5.3	798
Solution.....	4.77	1.6	
Tissue.....		6.9	792
Solution.....	3.02	1.4	
Tissue.....		7.9	777

<i>Derived Data</i>						
	pH	pCO <sub>2</sub>	[HCO <sub>3</sub> <sup>-</sup> ]	$\frac{[\text{HCO}_3^-]_C}{[\text{HCO}_3^-]_E}$	E	(H <sub>2</sub> O)
	*	<i>mm.</i>	<i>m.eq. per kg. H<sub>2</sub>O</i>		<i>gm. per kg. muscle</i>	<i>gm. per kg. cells</i>
External solution.....	8.03	27.1	87.0			
Intracellular phase.....		27.1		*	328	700
External solution.....	7.78	40.2	70.2			
Intracellular phase.....	6.53	40.2	6.4	0.091	141	743
External solution.....	7.57	35.6	38.4			
Intracellular phase.....		35.6		*	150	740
External solution.....	7.43	25.2	19.6			
Intracellular phase.....	6.87	25.2	5.8	0.30	281	744
External solution.....	6.64	25.6	3.28			
Intracellular phase.....	6.94	25.6	6.81	2.08	242	739
External solution.....	5.59	28.0	0.3			
Intracellular phase.....	6.90	28.0	6.91	24.7	219	740
External solution.....	4.74	27.3	0.04			
Intracellular phase.....	7.05	27.3	9.50	237.0	193	745
External solution.....	3.02	25.0	0.00			
Intracellular phase.....	7.12	25.0	10.2		200	704

\* In these experiments, the calculated extracellular bicarbonate more than accounted for the bicarbonate in the whole tissue, leaving none for the fibers.





# THE RÔLE OF PYRUVATE IN THE METABOLISM OF ETHYL ALCOHOL

By W. W. WESTERFELD, ELMER STOTZ, AND ROBERT L. BERG

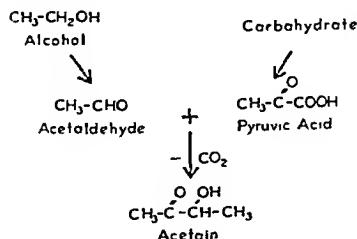
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(Received for publication, May 13, 1942)

There is a great deal of evidence that the metabolism of alcohol is favored by the simultaneous metabolism of carbohydrate. Thus, alcohol increases the blood sugar at the expense of liver glycogen (1-3), and when the latter is depleted by fasting, alcohol utilization is depressed (4-6) and parallels carbohydrate oxidation (7, 8). Feeding large doses of carbohydrate increases the rate of alcohol oxidation (8, 9). Insulin stimulates the metabolism of alcohol, especially when given simultaneously with sugar (10-15). Other evidence of a relationship between carbohydrate and alcohol metabolism has been obtained (15-20). One intermediary of carbohydrate metabolism, *i.e.* pyruvate, has been shown by Leloir and Munoz (6) to accelerate the oxidation of alcohol by liver slices.

The nature of this relationship has remained obscure because the complete pathway of alcohol metabolism is unknown. It is relatively certain that the first step is an oxidation of alcohol to acetaldehyde, though the evidence is indirect. The only known enzyme which will attack ethyl alcohol is the alcohol dehydrogenase (21-25), which converts it to acetaldehyde. This enzyme is located almost exclusively in the liver (6, 21, 23), and there is abundant evidence from perfusion studies (3, 5, 10, 26-28) and liver poisoning (5, 10, 29-31) that the liver is the principal organ in the body capable of the initial oxidation of alcohol.

The possibility that alcohol metabolism then proceeds by condensation of the acetaldehyde with pyruvate to form acetoin was suggested by the recent preparation from mammalian tissues by Green *et al.* (32) of the



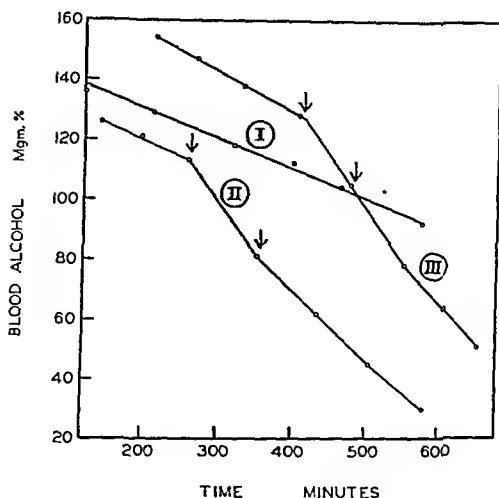


FIG. 1. The effect of administered pyruvate on the disappearance of blood alcohol. Dog 3M; 13.9 kilos. Alcohol was administered at 0 time, 30 cc. for Curves I and II, 35 cc. for Curve III. Control Curve I, no further treatment; rate of fall of blood alcohol, 6.0 mg. per cent per hour. Curve II, 10 gm. of sodium pyruvate in 125 cc. of water given orally at each arrow; control rate 6.6 mg. per cent per hour; after pyruvate 21.8. Curve III, 5 gm. of sodium pyruvate in 75 cc. of water given orally at each arrow; control rate 8.2 mg. per cent per hour; after pyruvate 21.2.

TABLE I  
*Effect of Administered Pyruvate on Disappearance of Blood Alcohol*

Dog and sex	Weight	Oral administrations		Time of pyruvate administration	Intercept at 0 time	Rate of decrease of blood alcohol per hr.		
		Absolute alcohol	Sodium pyruvate			Control	After pyruvate	
							1st hr.	2nd hr.
	kg.	cc.	gm.	min.	mg. per cent blood alcohol	mg. per cent	mg. per cent	mg. per cent
2F	9.8	25	5, 5	266	147	8.1	25.2	19.5
2F	9.8	30	10,* 10	404	202	8.1	27.2	17.8
4M	22.0	40	10, 5	405	140	7.7	11.3	10.9
5F	13.6	35	5, 5	409	174	8.4	16.0	15.7
6M	12.3	35	5, 5	376	190	9.5	25.3	22.2
Average†.....						8.1	21.1	17.4

\* 5 gm. of alloxan were administered with the first dose of pyruvate.

† The average includes experiments on Dog 3M (plotted in Fig. 1).

tions were offered (38, 39). Further studies by LeBreton (40) and Eggleton (41) left little doubt that alanine increased the rate of alcohol metabolism.

The conversion of alanine to pyruvate by oxidative deamination is a well known metabolic reaction, and the effect of alanine on the blood alcohol curve is attributed to the pyruvate formed. The increase in blood

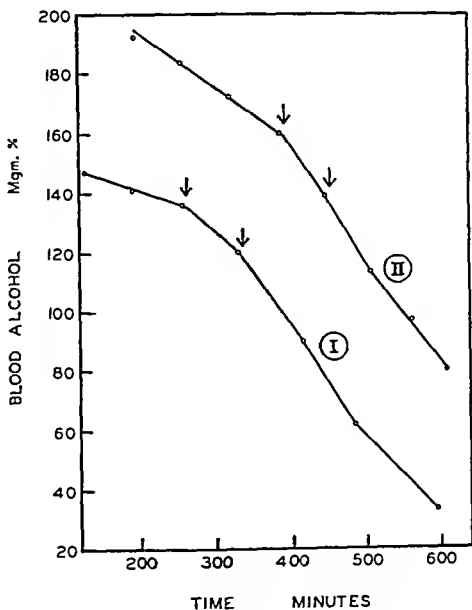


FIG. 2. The effect of *dl*-alanine on the disappearance of blood alcohol. Curve I, Dog 2F; 9.8 kilos. 25 cc. of alcohol were administered at 0 time. 8.5 gm. of alanine in 75 cc. of water given at each arrow. Control rate 5.0 mg. per cent per hour; after alanine 13.2 (1st hour), 21.8 (2nd hour). Curve II, Dog 7F; 18.4 kilos. 47.5 cc. of alcohol were administered at 0 time; 8.0 gm. of alanine in 125 cc. of water given at each arrow. Control rate 10.6 mg. per cent per hour; after alanine 22.2 (1st hour), 24.7 (2nd hour).

pyruvate and lactate resulting from alanine administration is illustrated in Fig. 3.

*Effect of Alcohol on Blood Pyruvate and Lactate Curves*—Comparison of the blood pyruvate and lactate curves resulting from the administration of pyruvate to untreated dogs and to dogs previously receiving alcohol showed an increased rate of pyruvate utilization during alcohol metabolism. In Fig. 4, Curves IP and IL trace the normal rise and fall in blood pyruvate and lactate respectively when 5 gm. of sodium pyruvate were given to a

fasted dog. When the pyruvate was given to the same dog  $7\frac{1}{2}$  hours after the usual dose of alcohol, Curves IIP and IIL were obtained. Although

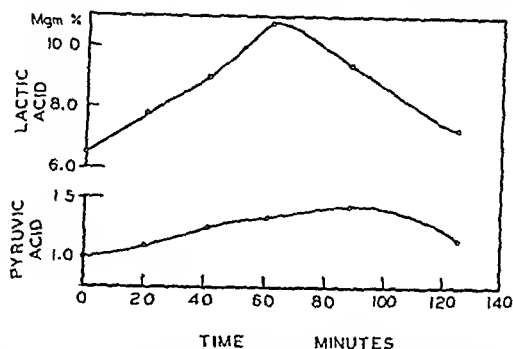


FIG. 3. Blood pyruvate and lactate response to the oral administration of *dl*-alanine. Dog 7F; 18.4 kilos. 8.0 gm. of alanine were given at 0 time.

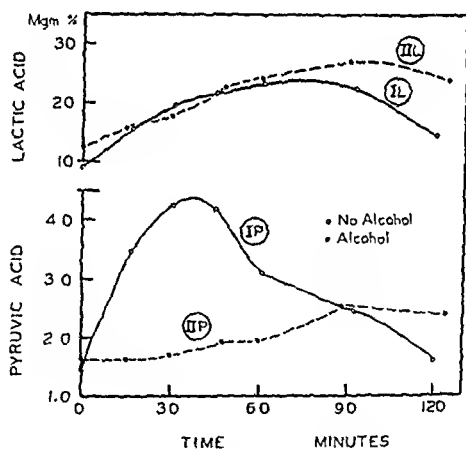


FIG. 4. Blood pyruvate and lactate response to the oral administration of sodium pyruvate (Curves I) normally and (Curves II) in the same dog having previously received alcohol. Dog 3M; 13.9 kilos. 5.0 gm. of sodium pyruvate in 75 cc. of water were given at 0 time; Curves IP and IL, blood pyruvate and lactate respectively in previously untreated dog. Curves IIP and IIL, the same when the pyruvate was given 452 minutes after the dog had received 35 cc. of alcohol.

the blood lactate curves are similar, the blood pyruvate rise in the dog metabolizing alcohol was markedly less than in the normal dog; during alcohol metabolism, pyruvate was utilized so rapidly that it failed to in-

crease appreciably the pyruvate concentration in the systemic veins. Thus, there exists a reciprocal relationship between pyruvate and alcohol metabolism, and the effect of pyruvate on the disappearance of alcohol is correlated with a simultaneous metabolism of the pyruvate.

Stotz and Bessey (42) found a fixed parallel relationship between the blood pyruvate and lactate in a normal animal, in spite of fluctuations in absolute amounts produced by factors such as anoxia and exercise. This ratio was found to be normal after pyruvate administration to the untreated dog, but in the dog metabolizing alcohol the ratio indicated an abnormally rapid removal of pyruvate. In thiamine deficiency, an abnormally slow removal of pyruvate was indicated by a ratio altered in the opposite direction (42).

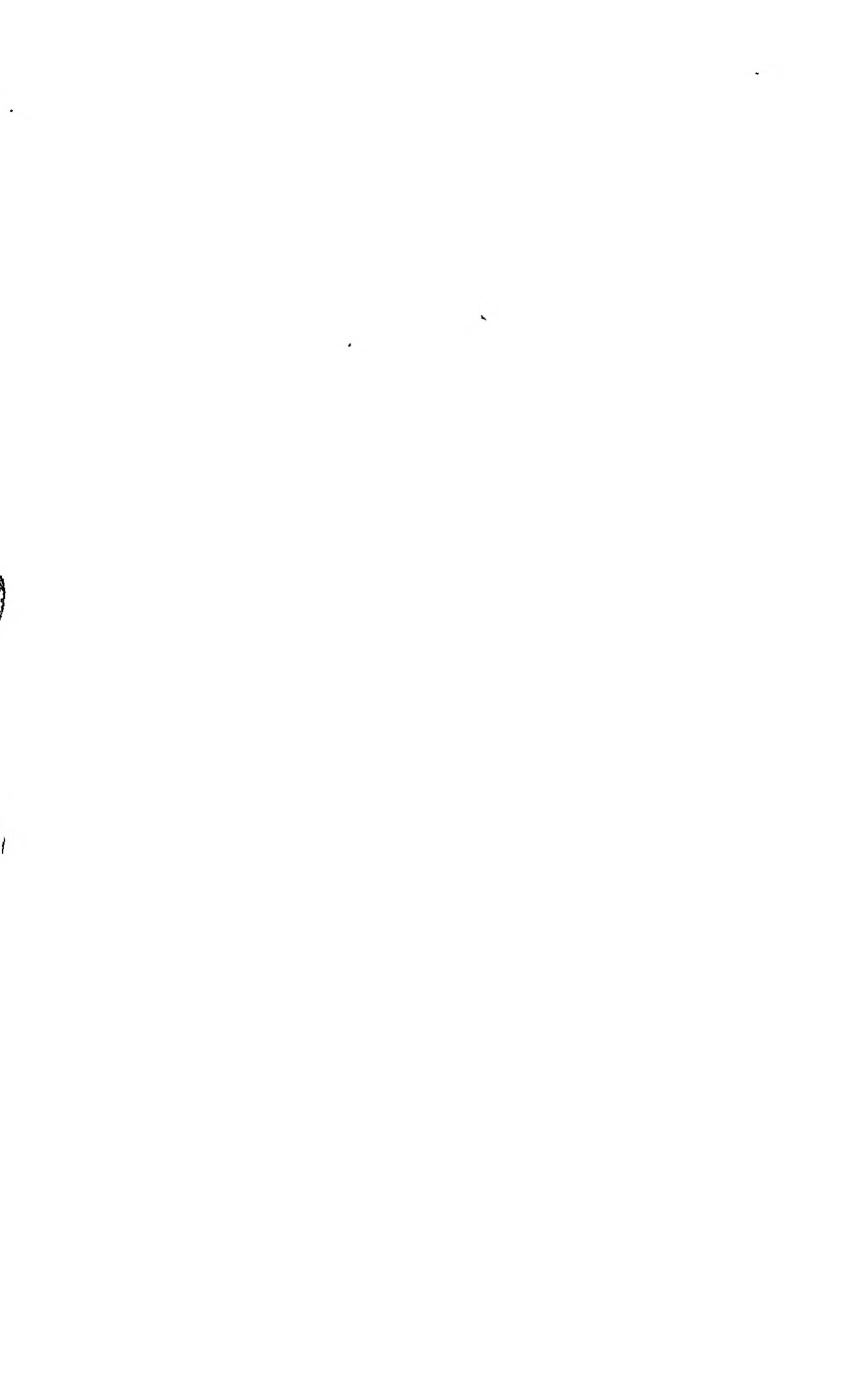
#### DISCUSSION

The *in vivo* relationship between pyruvate and alcohol metabolism demonstrated in this study provides a rational basis for the various facts recorded in the literature concerning the relationship between carbohydrate and alcohol metabolism. The increased alcohol oxidation resulting from insulin may also be due to an increased pyruvate production, especially since insulin has been shown (43) to increase the formation of pyruvate from glucose. Widmark (13) showed that insulin was unable to increase the rate of alcohol metabolism in dogs beyond 19 mg. per cent per hour.

Leloir and Munoz (6) previously reported that pyruvate increased the oxidation of alcohol by liver slices, and attributed this effect to a coupled oxidation-reduction of the alcohol and pyruvate. The evidence obtained in the present study cannot exclude this possibility as an explanation for the pyruvate effect, nor can the *in vitro* studies be entirely explained by the oxidation-reduction mechanism. The need for thiamine in the metabolism of alcohol (44) tends to support the acetaldehyde-pyruvate condensation mechanism, but the establishment of either one or a combination of both of these theories as the mechanism of the pyruvate effect awaits further study.

The so called carbohydrate sparing action of alcohol (45-51), which appears to be diametrically opposed to the previously cited fact that alcohol requires simultaneous carbohydrate oxidation, may be reconciled on the basis of the proposed mechanism. In normal carbohydrate metabolism, 2 moles of pyruvate would be required to form 1 mole of acetoin, whereas during alcohol metabolism, only 1 mole of pyruvate (from carbohydrate) and 1 mole of acetaldehyde (from alcohol) would be required to form the mole of acetoin.

A metabolic basis for the occurrence of "alcoholic" pellagra (52, 53) and polyneuritis (44, 54-57) in chronic alcoholics is furnished by the proposed pathway, since the oxidation of alcohol to acetaldehyde requires



# PROTEINS OF HUMAN SEMINAL PLASMA\*

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There have been few recent investigations dealing with the proteins of human seminal plasma. In 1888 Posner (1) found an unusual protein in urine, traced its presence to contamination with semen, and identified it as a proteose (propeptone). Slowtsoff (2) reported the presence of "nuclein," but it is not clear whether he worked with seminal plasma or whole semen, though the latter appears to be more likely. He also found proteoses, as well as albumin and traces of mucin. Fürbringer (3) stated that the seminal vesicles contain a gelatinous secretion which liquefies soon after seminal ejaculation and which he believed to be a globulin. We have been unable to find a description of the experiments upon which this belief was based. Recently, Goldblatt (4) reported that seminal plasma contains primary and secondary proteoses, a considerable amount of albumin, a small quantity of nucleoprotein, and traces of globulin and mucin. He states that mucin is removed from semen by centrifugation.

Our purpose was to investigate the proteins of human seminal plasma, by means of electrophoretic, chemical, and serologic methods. The present report records the results of electrophoretic examination of the plasma and of certain fractions obtained by chemical treatment. In the course of this work three protein components have been separated in an electrophoretically pure form. The serologic experiments will be reported elsewhere later.

## Methods

Semen specimens were obtained from apparently healthy men and were centrifuged as promptly as possible. The electrophoretic analyses were carried out by use of the Tiselius apparatus (5) having a single section long micro cell (capacity 2 ml.) and the Toepler schlieren optical method with adaptations of Longsworth (6) and of Philpot (7) and Svensson (8). The channel of this cell is 2 mm. wide, 15 mm. deep (along the optic path), and 50 mm. high. The bottom connecting link completing the U-tube has the same area as the upright limbs and, together with one upright limb, is filled with the seminal plasma. The rest of the system is filled

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with buffer. Protein-buffer boundaries were formed by realigning the central section of the cell with bottom and top connecting channels by means of a pneumatic piston. Before voltage was applied, the boundaries were pushed into full view by means of a clock and plunger, arranged to move the boundary 4 mm. in 25 minutes. This was slow enough to avoid disturbing the boundaries, which were then photographed to give a starting point for mobility measurements. This arrangement has been used to great advantage in studying materials obtainable in limited quantities, such as cerebrospinal fluid (9), and in identifying separated serum components (10). The mobilities and character of patterns obtained with this apparatus agree exactly with those obtained with the standard electrophoresis cell of 11 ml. capacity.

The preparations were dialyzed in viscose sausage casings in the refrigerator against 500 cc. of phosphate buffer containing 0.055 M NaCl (pH 7.85 and ionic strength 0.1) for 24 hours and then against a liter of fresh buffer for 24 hours. The second volume of buffer was used as the electrode solution. Electrophoresis was carried out at about 1.5°. The current employed was usually 9 milliamperes, giving a potential in the cell of about 6.4 volts per cm. Conductance (11) and pH measurements were made on the buffer solution in every experiment and with few exceptions on the preparation also. The mobilities are expressed in  $\text{cm.}^2 \text{ volt}^{-1} \text{ sec.}^{-1} \times 10^{-5}$ . The chemical treatment will be described separately for each preparation.

### *Observations and Interpretations*

Several electrophoretic patterns of whole seminal plasma are shown in order to illustrate quantitative differences in the composition of different specimens. These differences arise from the fact that the plasma as a whole is composed of fluids contributed by several organs.

The electrophoretic patterns of two samples of seminal plasma are shown in Patterns A1 and A2, Fig. 1. In both there are four distinct peaks, referred to in this article as P1, P2, P3, and P4, in the order of increasing mobility. One of these had not moved at the pH at which the experiment was done. In a third pattern, No. A3, on a pooled sample which was dialyzed much longer than usual a fifth component is indicated. It is the most rapidly moving component and is seen in some of the patterns of certain fractions of seminal plasma to be described later. Pattern A4<sup>1</sup> was

<sup>1</sup> The semens from which the plasmas for Patterns A1, A2, and A4 were taken were examined microscopically, and were found to contain whole sperm in normal numbers. Morphologically, a very large percentage of the cells in the first two was normal; in the last a rather high percentage of abnormal cells was present, although two earlier specimens from the same individual showed relatively few abnormal cells.

obtained from a single specimen of plasma and illustrates a relative deficiency of components P3 and P4.

To aid in the identification of the proteins represented by the different peaks in the patterns of whole seminal plasma, several procedures were used.

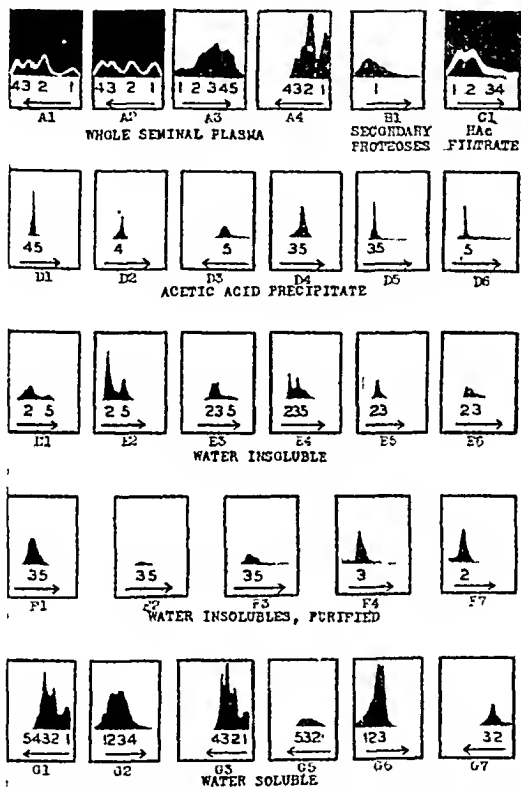


FIG. 1. Electrophoretic patterns of seminal plasma and fractions thereof. The numbers immediately under the patterns refer to the protein components indicated above them. Patterns with arrows pointing left are ascending; right, descending. The numbers under each block are electrophoretic pattern numbers.

The "proteose" fraction passed through the viscose membrane and was not coagulated by heat. Several plasmas were dialyzed against small volumes of buffer solution which were pooled and half saturated with ammonium sulfate. The supernatant of this was then completely saturated with the same salt. The first fraction gave precipitates from weakly acid solutions with potassium ferrocyanide, sodium tungstate, and potassium picrate.

Addition of 10 per cent  $\text{HNO}_3$  yielded no precipitate; subsequent saturation with NaCl produced a moderate precipitate. Saturation with NaCl alone produced only a small precipitate after 2 days. Strong ammonia did not precipitate the protein. The biuret and xanthoproteic tests were positive, the Millon test faintly so.

The second fraction also was precipitated, after the addition of acetic acid, by potassium ferrocyanide and sodium tungstate but not by potassium picrate nor by strong ammonia. As with the first fraction,  $\text{HNO}_3$  caused no change until after saturation with NaCl. Saturation with NaCl caused no precipitation unless acetic acid was present. The protein color tests gave the same results as described above.

An electrophoretic pattern of the isolated second fraction is shown in Pattern B1. The mobility 0.9<sub>6</sub> identifies this fraction with peak P1 in the patterns of whole plasma.<sup>2</sup> There is indication in this pattern that more than one molecular species is present in this fraction. The varying values calculated for the mobility of this slowest moving component from several patterns in which it appears (Table I) support this idea.

To some diluted seminal plasma, 1 per cent acetic acid was added. The precipitate which collected after 18 hours was removed and the supernatant was examined electrophoretically. Peaks P1 and P2 in Pattern C1 show clearly, and there are traces of the faster moving components. The experiment was repeated on two pooled specimens. Peaks P1 and P2 in Pattern C2, not shown in Fig. 1, were again present and peak P3 was just visible as an inflection in the P2 peak, more noticeable in the picture of the ascending limb than in that of the descending one. Component P4 was completely removed.

The acetic acid precipitate was then investigated. Two specimens of plasma were pooled and dialyzed against running water. After the removal of the precipitate which formed, enough 5 per cent acetic acid was added to the supernatant to give maximal precipitation, and the precipitate so obtained was dissolved in 0.05 M HCl and reprecipitated by the addition of several volumes of water. It was then dissolved in the buffer solution and submitted to electrophoretic analysis. Its pattern, No. D1, shows a main component with a mobility of 6.3<sub>8</sub> (P5) and a second one with a mobility of 5.5<sub>8</sub> (P4). From another specimen of plasma, not dialyzed against water, but diluted with 4½ volumes of water and then treated with acetic acid, a precipitate was likewise obtained. This was dissolved in the buffer solution with the aid of a little NaOH. The electrophoretic pattern, No. D2, shows a single component with a mobility of 5.5<sub>6</sub> which corresponds with that of P4 in the patterns of whole plasma. There is no peak for P5 even after 1½ hours.

<sup>2</sup> It has been observed that this component moves noticeably only in the descending limb of the apparatus at pH 7.85.

TABLE I

*Electrophoretic Mobilities of Seminal Plasma Proteins*

Mobilities are expressed as  $\text{cm}^2 \text{ volt}^{-1} \text{ sec}^{-1} \times 10^{-5}$  Phosphate buffer pH 7.85, containing 0.055 M NaCl; total ionic strength 0.1; potential 6.4 volts per cm

Pattern No	Ascending or descending	Contents of cell	Components*				
			P1	P2	P3	P4	P5
A1	A.	Whole seminal plasma	$\pm 0$	2.8 <sub>3</sub>	4.2 <sub>4</sub>	5.4 <sub>6</sub>	—
A2	"	" " "	$\pm 0$	2.3 <sub>3</sub>	4.4 <sub>2</sub>	5.3 <sub>5</sub>	—
A3	D.	" " " (pooled)	0.5 <sub>4</sub>	3.2 <sub>5</sub>	4.4 <sub>1</sub>	5.9 <sub>4</sub>	6.4 <sub>8</sub>
A4	A.	" " "	$\pm 0$	2.9 <sub>3</sub>	4.5 <sub>0</sub>	5.6 <sub>9</sub>	—
B1	D.	Secondary proteoses	0.9 <sub>6</sub>	—	—	—	—
C1	"	Filtrate from acetic acid-treated plasma	1.4 <sub>5</sub>	3.1 <sub>4</sub>	Trace	Trace	Trace
C2	A.	" " "	$\pm 0$	3.3	4.5	—	—
D1	D.	Acetic acid precipitate	—	—	—	5.5 <sub>8</sub>	6.3 <sub>8</sub>
D2	"	" " "	—	—	—	5.5 <sub>0</sub>	—
D3	A.	" " " (pooled)	—	—	—	—	6.2 <sub>5</sub>
D4	D.	" " "	—	—	4.9 <sub>4</sub>	—	6.0 <sub>3</sub>
D5	"	" " "	—	—	5.0 <sub>8</sub>	—	6.4 <sub>3</sub>
D6	"	" " " (pooled)	—	—	—	—	6.5 <sub>9</sub>
D7	A.	" " "	—	—	4.7 <sub>3</sub>	5.7 <sub>8</sub>	6.1 <sub>2</sub>
E1	D	Water-insoluble fraction	—	2.5 <sub>3</sub>	—	—	6.1 <sub>1</sub>
E2	"	" " "	—	2.7 <sub>8</sub>	—	—	6.1 <sub>9</sub>
E3	"	" " "	—	3.0 <sub>8</sub>	4.1 <sub>3</sub>	—	6.3 <sub>9</sub>
		(pooled)					
E4	"	Water-insoluble fraction	—	2.9 <sub>3</sub>	4.9 <sub>0</sub>	—	6.2 <sub>0</sub>
E5	"	" " "	—	2.7 <sub>2</sub>	4.3 <sub>4</sub>	—	—
E6	"	" " "	—	3.0 <sub>8</sub>	4.4 <sub>3</sub>	?	—
F1	"	" " " partly purified	—	—	4.5 <sub>8</sub>	—	6.0 <sub>8</sub>
F2	"	" " "	—	—	4.7 <sub>8</sub>	—	?
F3	"	" " "	—	—	4.9 <sub>5</sub>	—	6.6 <sub>4</sub>
F4	"	Water-insoluble P3†	—	—	3.8 <sub>2</sub>	—	—
F5	"	" " "	—	—	3.6 <sub>2</sub>	—	—
F6	"	" " "	—	—	3.9 <sub>5</sub>	—	—
F7	"	" " P2	—	2.8 <sub>9</sub>	—	—	—
G1	A.	Water-soluble fraction	0.1 <sub>7</sub>	2.6 <sub>7</sub>	4.4 <sub>1</sub>	5.6 <sub>4</sub>	6.2 <sub>1</sub>
G2	D.	" " "	1.2 <sub>2</sub>	3.0 <sub>5</sub>	4.5 <sub>8</sub>	5.7 <sub>9</sub>	—
G3	A.	" " "	$\pm 0$	2.7 <sub>0</sub>	4.6 <sub>8</sub>	5.8 <sub>2</sub>	—
G4	D.	" " "	0.7 <sub>1</sub>	2.5 <sub>6</sub>	4.6 <sub>9</sub>	5.5 <sub>8</sub>	—
G5	A	" " " (precipitate obtained by two-thirds saturation with ammonium sulfate)	—	3.0 <sub>4</sub>	4.7 <sub>7</sub>	—	6.3 <sub>4</sub>
G6	D	Water-soluble fraction (mucin removed with acetic acid)	1.3 <sub>7</sub>	2.8 <sub>5</sub>	3.7 <sub>9</sub>	—	—
G7	A.	" " "	—	3.0 <sub>5</sub>	4.2 <sub>6</sub>	—	—
Average				2.8 <sub>9</sub>	4.5 <sub>9</sub> †	5.6 <sub>5</sub>	6.3 <sub>0</sub>

Addition of 10 per cent  $\text{HNO}_3$  yielded no precipitate; subsequent saturation with NaCl produced a moderate precipitate. Saturation with NaCl alone produced only a small precipitate after 2 days. Strong ammonia did not precipitate the protein. The biuret and xanthoproteic tests were positive, the Millon test faintly so.

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showed lower mobilities which lie between the mobilities of P3 (obtained at pH 4.5 to 5.5) and of the precipitate obtained at pH 7.2, suggesting that the two peaks might have fused in these patterns. The whole plasma Pattern A1 (ascending) shows inflection in the P3 peak at a point which would fit the supposition that there is a component with a mobility intermediate between those of P2 and P3. The same is true in Patterns A2 (ascending, 2 hours), A3 (descending, 2 hours), and A4 (ascending, 2 hours, not illustrated in Fig. 1). The evidence suggests that the protein precipitated at pH 7.2 is not P3. We have nevertheless listed it as P3 in Table I.

After the removal of the protein just described, more alkali was added. An additional precipitate formed, the pattern of which is shown in Pattern F7; the mobility is 2.8<sub>s</sub>. This is concluded to be water-insoluble P2.

Patterns G1, G2, and G3 of the material in solution following removal of the precipitate formed on dialysis of seminal plasma against water resemble those of whole seminal plasma. The mobilities correspond with those calculated for the several components in whole plasma. Since there are two components with the same mobilities as those of water-insoluble P2 and P3 and since extended dialysis does not precipitate these, it appears that the two water-insoluble proteins have water-soluble analogues. Some of these patterns suggest that there is a component with a mobility intermediate between those of P2 and P3. The pattern of the material precipitated out of the water-soluble fraction by two-thirds saturation with ammonium sulfate is shown in Pattern G5. Only the proteose P1 remained in solution.

On addition of acetic acid to the water-soluble fraction, mucin is precipitated and patterns of such precipitates have already been discussed. Two patterns of the supernatant of the acetic acid precipitate are shown in Patterns G6 and G7. P2 and P3 remain in solution.<sup>4</sup> We have not yet attempted to separate water-soluble P2 and P3 as was done for the water-insoluble P2 and P3.

#### DISCUSSION

Previous investigators have reported the presence of a trace of albumin. Goldblatt (4), however, concludes that there is a considerable amount, because he believes that centrifugation removes the mucin from seminal plasma and because heating centrifuged, acetic acid-treated specimens yields a large amount of coagulum. We have observed the presence of mucin in all plasmas even after several hours of centrifugation. The electrophoretic patterns support the opinion that there cannot be more than 0.02 per cent albumin present, for the only components moving with approximately the same mobility as albumin are P4 and P5. Both of these

<sup>4</sup> In Pattern G6 the component listed under P3 may be a water-soluble analogue of the water-insoluble protein listed in the same column in Patterns F4, F5, and F6.

are precipitated by acetic acid, and following their removal the filtrate contains little or no material with the mobility of albumin. The apparatus is capable of detecting about 0.02 per cent.

Fresh specimens of seminal plasma in which cell autolysis has not occurred appear to contain only minute quantities of nucleoprotein. The presence of 0.15 per cent phosphorus in one of the acetic acid precipitates could be due to the presence of a small amount of nucleoprotein in this material. However, mucins are known to adsorb and hold inorganic salts strongly, and, since phosphates are present in seminal plasma in relatively high concentration, some of the phosphorus found might have come from this source. In 4.5 cc. of a certain sample of seminal plasma 0.01 mg. of organic phosphorus was found. In this specimen there could have been, therefore, 0.04 per cent nucleoprotein with 0.5 per cent phosphorus content.

The mobilities of P2 and P3 components are similar to those of  $\beta$ - and  $\alpha$ -globulin of blood serum. Preliminary serological tests indicate that water-insoluble P3 is probably not identical with any of the blood serum proteins. Water-insoluble P2 has not been studied sufficiently to give its serological relationship to the blood proteins. On the other hand, the water-soluble material shown in Pattern G6 yielded a heavy precipitate with serum of rabbits immunized with human serum. This precipitate is considerably larger than the corresponding one obtained with anti-human seminal plasma serum. Further work is being done with the serological reactions of the seminal proteins.

Whereas fresh specimens of seminal plasma, when dialyzed against water, always yield a relatively considerable precipitate consisting largely of components P2 and P3, older specimens yield less, and specimens allowed to stand at room temperature for 5 days yield little or no precipitate. The cause of this change has not yet been investigated. It may be related to, or perhaps be a continuation of, the liquefaction process which sets in much more promptly.

#### SUMMARY

The proteins of human seminal plasma have been examined electrophoretically and some of them chemically as well. Non-heat-coagulable protein (protease), referred to as component P1, was found to pass through viscose membranes (average pore diameter 25 Å.). Of the protein which passed out of the bag, the fraction precipitable by full saturation with ammonium sulfate, but not by one-half saturation, had a mobility of  $-0.9$  cm.<sup>2</sup> volt<sup>-1</sup> sec.<sup>-1</sup>  $\times 10^{-5}$  in phosphate buffer of pH 7.85 and ionic strength 0.1 when measured in the descending limb of the cell. There appears to be more than one molecular species present in this fraction, as judged by the electrophoretic pattern.

A glycoprotein, component P4, has been chemically separated in an electrophoretically homogeneous state, though irregularly. It has a mobility of  $-5.6_s$ , contains 9.3 per cent nitrogen, and yields 26.8 per cent reducing substances (as glucose) following treatment with  $\text{NHCl}$  at  $100^\circ$ . It contains 10.8 per cent hexosamine and no uronic acid.

Two water-insoluble proteins, P2 and P3, have been prepared, the former electrophoretically pure. They have two water-soluble analogues. Their mobilities are  $-2.8_s$  and  $-4.5_s$  respectively (averages of water-insoluble and water-soluble values in whole seminal plasma and in fractions of it). A water-insoluble component with a mobility of  $-3.8_s$  has been separated in an electrophoretically homogeneous state. It is not yet clear whether this is identical with P3, possessing a lower mobility because it is free of any other seminal protein, or whether it is a discrete protein. The latter seems more likely.

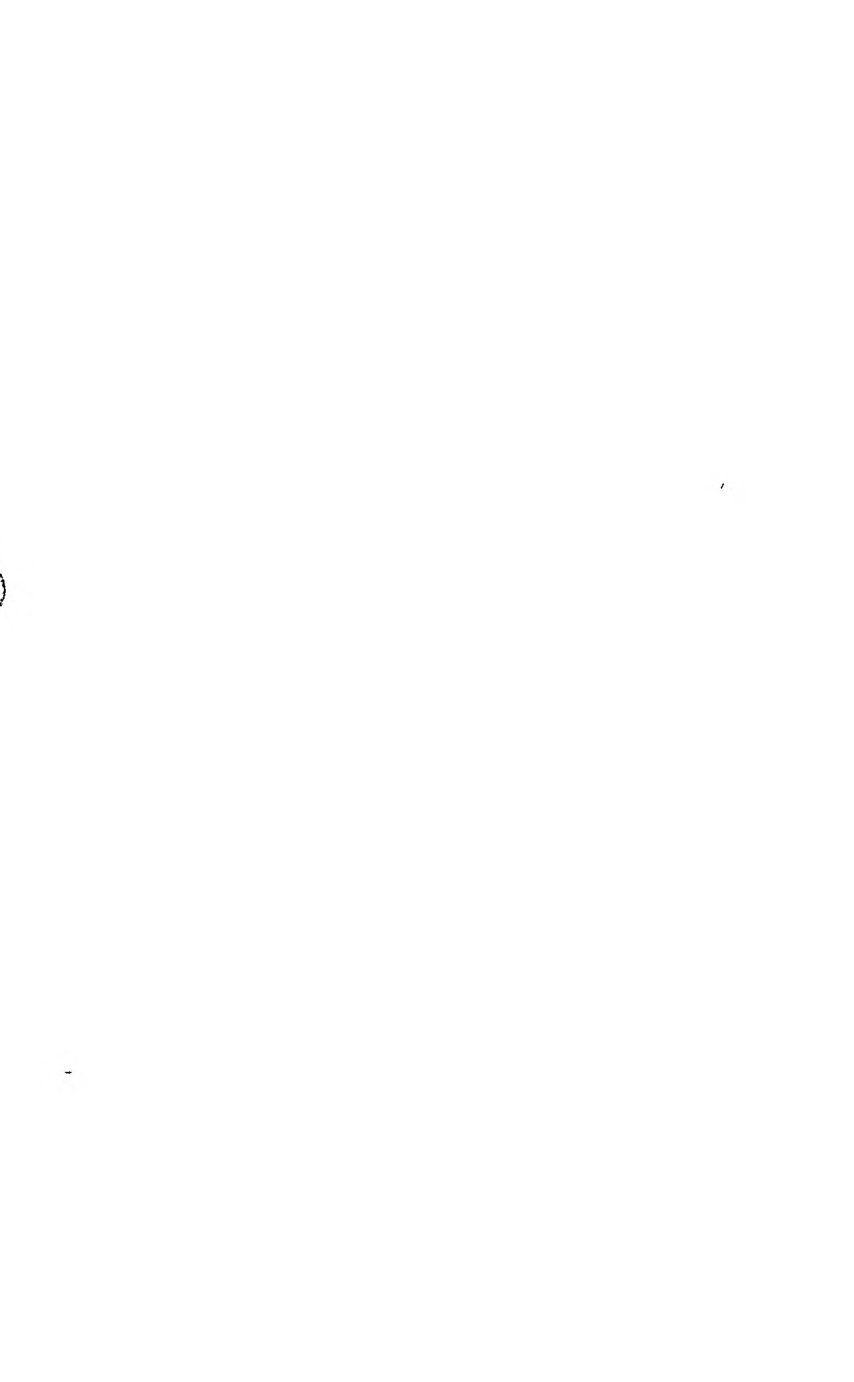
The electrophoretic evidence indicates that there is probably less than 0.02 per cent albumin present in seminal plasma.

Chemical evidence indicates that there is probably less than 0.04 per cent nucleoprotein present.

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# NICOTINIC ACID DEFICIENCY STUDIES IN DOGS\*

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The importance of nicotinic acid in the nutrition of the dog was demonstrated through the use of a modified Goldberger diet (1). Since that time studies on other members of the B complex in the nutrition of the dog have been carried out by using a purified casein-sucrose ration supplemented with synthetic vitamins. Dogs may be maintained with good growth over rather long periods of time when this ration is supplemented with thiamine, riboflavin, nicotinic acid, pyridoxine, pantothenic acid, and choline (2). In this paper we wish to describe the results obtained when nicotinic acid is omitted from this ration and give data for the nicotinic acid requirement of dogs, as well as assays on a few food materials. West (3) demonstrated that sulfapyridine inhibits the curative action of nicotinic acid in dogs on a Goldberger diet. Results will be presented to show that similar effects are obtained when the purified synthetic ration is used.

## EXPERIMENTAL

Weanling mongrel puppies and older growing dogs were used in these studies. The basal ration has been described previously (4) and consists of sucrose 66 per cent, acid-washed casein 19 per cent, cottonseed oil 8 per cent, cod liver oil 3 per cent, and salt mixture 4 per cent. This ration was fed *ad libitum* and each dog received in addition 100  $\gamma$  per kilo of body weight per day of thiamine and riboflavin, 60  $\gamma$  of pyridoxine hydrochloride, 500  $\gamma$  of calcium pantothenate, and 50 mg. of choline chloride. The required amount of each of the vitamins was given in aqueous solutions twice weekly.

Supplements of nicotinic acid or materials to be assayed were not given until the dogs showed significant loss of weight and the typical early symptoms of blacktongue. The dogs usually refused food for at least 2 days before therapy was administered, but in order to reduce the loss of dogs, the

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animals were treated before severe symptoms developed. The extent of the growth response and the time required for symptoms to reappear after administering known quantities were very consistent in each individual dog. Three or four standard doses of nicotinic acid were used to standardize each dog and the gain in gm. of body weight per mg. of nicotinic acid administered was calculated in each case. Each dog was used for numerous assays and in one case eighteen assays were completed. The daily requirement per kilo of body weight was determined by using the average body weight during the assay period.

A typical example of the results obtained with a single animal is shown in Fig. 1. Dog 189 placed on the nicotinic acid-free ration as a weanling puppy exhibited deficiency symptoms characterized by drastic loss in

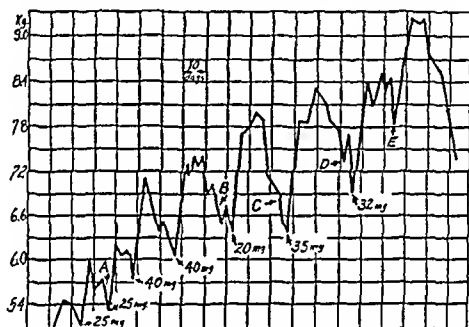


FIG. 1. Growth response of Dog 189 to various levels of nicotinic acid and other supplements. The figures indicate the level of nicotinic acid administered. A, 52 mg. of 2-aminonicotinic acid; B, wheat assay, 500 gm.; C, milk started, 2100 cc.; D, milk started, 5600 cc.; E, milk started, 4000 cc.

weight, inflammation of the gums, and palatine redness in 14 days. Nicotinic acid at 25 and 40 mg. levels was then administered in order to obtain standard responses. From these trials it was calculated that 1 mg. of nicotinic acid produced 34 gm. of gain in body weight in this dog. It may be noted that 2-aminonicotinic acid was given after the first dose of nicotinic acid and no response whatsoever was observed. After standardization this dog was used for determining the nicotinic acid content of wheat and milk. When the dog was declining in weight after the second 40 mg. dose of nicotinic acid, 500 gm. of ground whole wheat were fed as a paste over a 4 day period. The loss of weight was retarded but no growth response was obtained. 20 mg. of nicotinic acid were administered on the 5th day. The response observed was greater than could be expected from the amount of added nicotinic acid and the excess response was equivalent

to about 27 mg. of nicotinic acid. Thus 500 gm. of the wheat contributed 27 mg. or contained 5.4 mg. per 100 gm. This sample of wheat was found to contain 5.5 mg. per 100 gm. by the micro biological method of Snell and Wright (5) with alkaline extraction.

This dog was used in four attempts to determine the nicotinic acid content of milk. The results varied greatly, for whenever a fairly severe deficiency was allowed to develop, the administration of milk would fail to bring the dog back on food or prevent the severe weight loss. In these cases it was finally necessary to administer nicotinic acid. If we calculate the nicotinic acid content from the degree of response at points *C*, *D*, and *E* on the curve, we obtain values of 9.5, 2.6, and 9.5 mg. per liter of milk. The lower values correspond in general with the more severe weight loss before administration of the milk. When other dogs were used, values averaging 4 mg. per liter of milk were obtained.

We felt that these variable results could be related to the effect of milk solids on the synthesis of nicotinic acid by the bacterial flora of the intestinal tract. A 24 hour urine sample was collected from Dog 189 a day before the 4 liters of milk were given at point *E* on the graph and the nicotinic acid content as determined by the micro biological method of Snell and Wright (5) was 0.2 mg. The urine sample collected from the dog after the milk was given contained 1.1 mg. Thus there was a significant increase in nicotinic acid excretion especially if we consider the fact that Sarett (6) has shown that the trigonelline excretion exceeds that of nicotinic acid. This fact may have little effect under these conditions, since Sarett (6) has also shown that it usually takes several days for the trigonelline excretion to increase after administration of nicotinic acid. According to bacterial assay the 4 liters of milk contained 3.2 mg. Since almost one-third of this amount appeared in the urine after 1 day and the dog showed considerable growth, synthesis of nicotinic acid must have taken place either in the tract or in the body tissues.

In order to determine whether the lactose in the milk had any effect on the synthesis, a weanling puppy was placed on the basal ration in which the sucrose was replaced by lactose. Deficiency symptoms appeared in 14 days. After two deficiency and treatment periods the lactose was replaced by sucrose and periodic deficiencies were produced. A litter mate placed on the sucrose diet developed the typical symptoms in 18 days. Two responses with 12 and 14 mg. of nicotinic acid were obtained and then the sucrose was replaced by lactose. With lactose in the ration severe deficiency states have been produced with subsequent responses to dosages of nicotinic acid varying from 17 to 52 mg. A study of these responses indicates that the requirement per kilo of body weight was slightly higher on the lactose ration than on the sucrose ration. Thus the effect of the milk must

be related to specific precursors of nicotinic acid or to the protein content of the milk.

A careful survey of the records of all the dogs used during the past 2 years shows that rather accurate values can be calculated for the nicotinic acid requirement of seven of the dogs. Values for four adult dogs and three growing dogs are given in Table I.

TABLE I  
*Nicotinic Acid Requirement of Dogs*

Dog No.	Litter	Age	Micrograms per kilo body weight per day
156	A	Adult	220
175	B	"	225
205	C	"	210
216	D	"	200
189	A	Growing	365
222	E	"	350
223	"	"	250

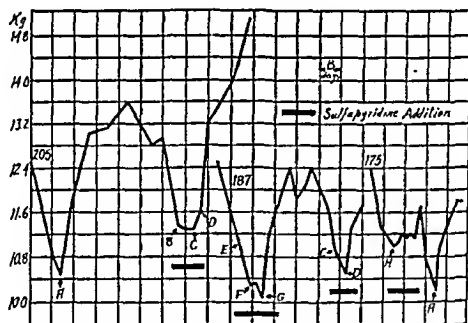


FIG. 2. Growth of nicotinic acid-deficient dogs receiving sulfapyridine. A, 40 mg. of nicotinic acid; B, 63 mg. of nicotinamide; C, dried whole liver = 150 gm. of fresh liver; D, 150 gm. of fresh liver; E, 113 mg. of nicotinic acid; F, 20 gm. of 1:20 liver extract powder; G, 115 gm. of fresh liver; H, 45 mg. of nicotinic acid. The figures on the curves represent the dog numbers.

**Sulfapyridine Inhibition**—When dogs showed definite nicotinic acid deficiency symptoms, sodium sulfapyridine monohydrate was administered orally in water solution at 8.00 a.m., 4.00 p.m., and 12.00 p.m., in doses sufficient to maintain a blood level of 5 to 10 mg. per 100 cc.; 2.5 gm. at each feeding were usually sufficient. Nicotinic acid and various liver supplements were given 24 to 36 hours after the sulfapyridine therapy was

started. A few of the results are shown in Fig. 2. After saturation with sulfapyridine, the addition of nicotinic acid, nicotinamide, dried whole liver, or liver extract 1:20 produced rather rapid alleviation of inflammation of the gums, palatine redness, and excessive saliva production, but the weight loss and the anorexia usually continued. Dog 205 gained 3.2 kilos in body weight when 40 mg. of nicotinic acid were given before saturation of the dog with sulfapyridine. After sulfapyridine feeding 63 mg. of nicotinamide failed to produce an increase in weight, dried liver gave a slight increase of 350 gm. in 3 days, but the feeding of an equivalent amount of fresh liver produced an increase of 1.1 kilos in 2 days and a total weight increase of 3.4 kilos in 17 days. Dog 175 when fed 45 mg. of nicotinic acid in the presence of the drug grew very slowly and finally gained 700 gm. The same dog showed a response of 1400 gm. when given 40 mg. of nicotinic acid under standard conditions. Dog 187 continued to lose weight after the administration of 113 mg. of nicotinic acid in addition to the drug. When 20 gm. of liver extract were fed, an additional loss of 250 gm. in body weight resulted. A growth response of 2.4 kilos was obtained when 115 gm. of fresh liver were fed. After another depletion dried liver failed to produce a weight gain, but fresh liver restored the dog's appetite and produced a gain of 1.2 kilos in body weight.

Sulfanilamide was used in place of sulfapyridine in Dog 216. Without sulfanilamide 48 mg. of nicotinic acid produced a response of 2.3 kilos, and a time interval of 30 days elapsed before symptoms reappeared. With sulfanilamide 50 mg. of nicotinic acid produced a response of 1.5 kilos and the symptoms reappeared in 10 days. Administration of whole fresh liver in the presence of sulfanilamide allowed an immediate growth response of 1.7 kilos.

#### DISCUSSION

Typical blacktongue symptoms such as drastic loss in weight, anorexia, inflammation of the gums, and palatine redness have been produced in dogs by feeding a synthetic diet containing all the available B vitamins except nicotinic acid. In weanling growing puppies, these symptoms appeared in 14 to 18 days. Adult dogs that had received the synthetic ration supplemented with various liver fractions before being shifted to the basal ration did not develop the deficiency until a period of 30 to 45 days had elapsed. Two dogs that had received commercial canned dog food for 8 months before being placed on the synthetic basal ration exhibited symptoms in 44 and 72 days respectively.

This basal ration appears to be more reliable than the modified Goldberger ration for nicotinic acid assays. Individual dogs can be standardized to definite quantities of nicotinic acid and as many as eighteen assays

may be obtained with one dog. Difficulties encountered in assaying foods such as milk are pointed out.

The nicotinic acid requirement for adult dogs ranges from 200 to 225  $\gamma$  per kilo of body weight. The requirement for the growing dog is 50 to 75 per cent higher, as one would expect. The values for the adult dogs may be of some use in calculating the minimum requirement for humans. Our adult dogs weighed about 10 kilos and if we assume the nicotinic acid requirement to follow the energy requirement at different body weights the requirement for a 10 kilo dog would be 49/75 of 225  $\gamma$  or 147  $\gamma$  if we use the energy values taken from the table of Brody, Proctor, and Ashworth (7). Thus the minimum requirement for a 70 kilo man would be approximately 10 mg. per day.

The interesting observation of West that sulfapyridine inhibits the curative action of nicotinic acid in dogs, and that fresh liver counteracts this inhibition, has been verified; however, it is improbable that the fresh liver functions by supplying cozymase. It is, of course, possible that sulfapyridine acts independently of the nicotinic acid-cozymase mechanism and that sulfapyridine would inhibit growth responses in other deficiencies. However, we have no evidence for this possibility. It is more likely that sulfapyridine either prevents the formation of cozymase or prevents its utilization after its formation from the added nicotinic acid. Similar studies (Elvehjem, Teply, and Axelrod, unpublished) with *Lactobacillus arabinosus* indicate that sulfapyridine inhibits the utilization of the cozymase. The fresh liver therefore may supply a labile protein which has sufficient affinity for the sulfapyridine to prevent its action in inhibiting the cozymase-linked reactions.

#### SUMMARY

1. An uncomplicated nicotinic acid deficiency may be produced on our highly purified casein-sucrose ration supplemented with thiamine, riboflavin, pyridoxine, pantothenic acid, and choline.

2. This ration seems suitable for the assay of food materials for their nicotinic acid content.

3. The requirement of nicotinic acid, as calculated by single dose feedings, for adult dogs ranges from 200 to 225  $\gamma$  per kilo of body weight per day and for young growing puppies ranges from 250 to 365  $\gamma$ .

4. Sulfapyridine feeding inhibits the response of the nicotinic acid-deficient dog to nicotinic acid, nicotinamide, dried liver, and liver extract powder. This inhibition is overcome by fresh liver.

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# THE ISOELECTRIC POINTS OF THREONINE AND SOME RELATED COMPOUNDS

BY CARL S. VESTLING AND DONALD T. WARNER

(From the Division of Biochemistry, Noyes Laboratory of Chemistry, University of Illinois, Urbana)

(Received for publication, June 8, 1942)

In connection with attempts in this laboratory to increase the yields of threonine in isolations from various proteins, it recently became essential to determine its isoelectric point. During the time since the isolation and identification of threonine by Rose and coworkers (1) and its subsequent synthesis by West and Carter (2), the isoelectric point of the pure compound has not been reported. Employing an electrometric method similar to that described by Hitchcock (3) in recalculating Sørensen's data (4) for glycine, we have carried out determinations of the apparent dissociation constants and of the isoelectric points of *l*-threonine, *dl*-allothreonine, *dl*-O-methylthreonine, *dl*-O-methylallothreonine, and *dl*- $\alpha$ -amino-*n*-butyric acid. The last named compound has been studied by other workers and serves as a reference substance.

The introduction of a "negative" hydroxyl group in  $\alpha$ -amino-*n*-butyric acid should increase the degree of dissociation of either or both acid groups. The data to be reported in this paper indicate that the dissociation of the adjacent substituted ammonium group in the resulting  $\alpha$ -amino- $\beta$ -hydroxy-*n*-butyric acid molecule is most affected. (See Table II.) Further evidence in support of these results may be noted by comparing the dissociation constants of alanine (5) with those of serine (6) and by a similar comparison in the case of lysine and hydroxylysine (7). Czarnetsky and Schmidt (8) reported data for a compound postulated to be a hydroxy-aminobutyric acid which they isolated from casein by the procedure of Schryver and Buston (9). The identity of their preparation with a pure  $\alpha$ -amino- $\beta$ -hydroxy-*n*-butyric acid is unlikely, however, since the shifts in dissociation constants noted when their compound is compared with  $\alpha$ -amino-*n*-butyric acid are in the opposite direction to that cited above.

## EXPERIMENTAL

The procedure employed in these experiments was that outlined by Hitchcock (3) with several modifications. We have used the following values for activity coefficients and for the ionic product of water:  $\gamma_{H^+} = 0.84$  (7);  $\gamma_{OH^-} = 0.81$  (7);  $pK'_w$  at  $25^\circ = 13.895$  (10). In all experiments the ionic strength fell within the limits  $0.100 \pm 0.002$ .

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The pH values were obtained at 25° with a Beckman pH meter equipped with traveling electrodes (glass electrode and saturated calomel half cell). The instrument was carefully standardized before and during each set of measurements by use of a standard acetate buffer, pH 4.62 (10), such that 0.0950 M HCl gave a pH reading of 1.08. It is estimated that the pH values could be read within the limits  $\pm 0.015$  pH unit (equivalent to

TABLE I

Apparent Dissociation Constants of L-Threonine-NaCl Solutions at 25° ( $\mu = 0.1$ )

HCl titration							
0.0950 M HCl	A, HCl concentration	C, threonine concentration	pH	$-\text{Log } [\text{H}^+]$	$\frac{A - [\text{H}^+]}{C}$ , fraction as cation	$\left(\frac{\text{Log } C}{A - [\text{H}^+]} - 1\right)$	pK'
ml.	mm per l.	mm per l.					
10.00	95.00		1.08				
5.02	47.60	49.91	1.88	1.805	0.640	-0.250	2.13
3.37	38.24	59.74	2.16	2.085	0.503	-0.005	2.17
2.14	28.47	70.03	2.45	2.375	0.346	+0.277	2.17
1.25	18.99	80.00	2.75	2.675	0.211	+0.573	2.18
0.56 <sub>6</sub>	9.63	89.84	3.16	3.085	0.098	+0.964	2.20
0.28 <sub>1</sub>	5.05	94.70	3.47	3.395	0.049	+1.288	2.18
Average							2.17
NaOH titration							
0.0993 M NaOH	B, NaOH concentration	C, threonine concentration	pH	$-\text{Log } [\text{OH}^-]$	$\frac{B - [\text{OH}^-]}{C}$ , fraction as anion	$\left(\frac{\text{Log } C}{B - [\text{OH}^-]} - 1\right)$	pK'
ml.	mm per l.	mm per l.					
0.34	6.32	93.65	7.82	5.983	0.068	+1.137	8.96
0.60	10.64	89.29	8.12	5.683	0.119	+0.869	8.99
1.23 <sub>1</sub>	19.64	80.22	8.52	5.283	0.245	+0.489	9.01
2.14	29.76	70.04	8.89	4.913	0.425	+0.131	9.02
3.32	39.62	60.10	9.29	4.513	0.659	-0.286	9.00
Average							9.00

$\pm 0.001$  volt). Carefully calibrated volumetric equipment was used throughout.

In Table I the complete data for one experiment with L-threonine are presented. The rather close agreement of the pK' values calculated from individual experimental points seems to justify the procedure used. The experimental deviations encountered in the threonine data are typical of those observed with the related compounds. For each experimental point,

5 ml. of a solution prepared by dissolving sufficient amounts of the pure compound and NaCl in conductivity water to make the resulting solution exactly 0.1 M with respect to each component was used. (Molalities have been used only in estimating the range of ionic strength encountered in these experiments. They were calculated from molarities by assuming unit density for each solution.)

In Table II a summary of the data obtained and of some other pertinent data is given. The dissociation exponents for the threonine group of compounds exhibit only slight changes as a result of structural or space modifications. It can be seen that the  $pK'_1$  values decrease systematically from top to bottom within the group, while the  $pK'_2$  values show a sig-

TABLE II  
Summary of Dissociation Exponents and Isoelectric Points

Compound	$pK'_1$	$pK'_2$	$pI'$	Remarks
<i>l</i> -Threonine.....	2.17	9.00	5.59	
<i>dl</i> -Allothreonine.....	2.11	9.01	5.56	$\Delta pI' = -0.03$
<i>dl</i> -O-Methylthreonine.....	2.02	9.00	5.51	
<i>dl</i> -O-Methylallothreonine...	1.92	8.90	5.41	$\Delta pI' = -0.10$
<i>dl</i> - $\alpha$ -Amino- <i>n</i> -butyric acid..	2.27	9.68	5.98	$\alpha$ -Amino- <i>n</i> -butyric acid vs. threonine. $\Delta pK'_2 = 0.68$
<i>dl</i> -Alanine (5).....	2.34	9.87	6.1	$t = 25^\circ$
<i>dl</i> -Serine (6).....	2.21	9.15	5.7	$\Delta pK'_2 = 0.72$
			$pK'_3$	
<i>l</i> -Lysine (7).....	2.20	8.90	10.28	$t = 38^\circ$
<i>l</i> -Hydroxylysine (7).....	2.13	8.62	9.67	$\Delta pK'_2 = 0.61$

The figures in parentheses represent bibliographic references.

nificant deviation only in the case of *dl*-O-methylallothreonine. This latter figure was checked in order to make sure that the correct value had been obtained. Hence it can be seen that allothreonine exhibits a very slightly more acid isoelectric point than does threonine ( $\Delta pI' = -0.03$ ), while the O-methyl derivatives show a somewhat greater difference in the same direction ( $\Delta pI' = -0.10$ ).

The constants for *dl*- $\alpha$ -amino-*n*-butyric acid agree reasonably well with those of Smith, Taylor, and Smith (11), when the fact that their values are true thermodynamic constants at infinite dilution is taken into account. It can be noted that the principal effect of substitution of a hydroxyl group in  $\alpha$ -amino-*n*-butyric acid is to increase the dissociation of the adjacent substituted ammonium part of the molecule; thus in the case of  $\alpha$ -amino-*n*-butyric acid versus threonine,  $\Delta pK'_2 = 0.68$ . Similarly in the case of

alanine versus serine,  $\Delta pK'_2 = 0.72$ , while in the case of lysine versus hydroxylysine,  $\Delta pK'_3 = 0.61$ .

The analyses of the five compounds under investigation are reported below. Van Slyke amino nitrogen determinations were carried out with a 2 per cent KI-glacial acetic acid solution (12), since threonine gives high values in the usual Van Slyke procedure. *dl*-Allothreonine could not be analyzed even by this modified method; macro-Kjeldahl analyses are reported for it. The  $\alpha$ -amino-*n*-butyric acid (Eastman product) was analyzed by the unmodified Van Slyke procedure.

Compound	N calculated	N found
<i>l</i> -Threonine	11 76	11 70
<i>dl</i> -Allothreonine	11 76	11 82
<i>dl</i> -O-Methylthreonine	10 53	10 68
<i>dl</i> -O-Methylallothreonine	10 53	10 62
<i>dl</i> - $\alpha$ -Amino- <i>n</i> -butyric acid	13 59	13 49

The authors desire to express their thanks to Professor W. C. Rose for the natural *l*-threonine and to Professor H. E. Carter for the synthetic related compounds used in this investigation.

#### SUMMARY

The apparent dissociation constants and the isoelectric points of *l*-threonine, *dl*-allothreonine, *dl*-O-methylthreonine, *dl*-O-methylallothreonine, and *dl*- $\alpha$ -amino-*n*-butyric acid have been determined by electrometric titration at 25° and ionic strength 0.1.

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# THE SYNTHESIS OF PLASTEINS BY THE ACTION OF TRYPSIN AND PAPAIN ON DIGESTS OF INSULIN

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(Received for publication, June 1, 1942)

Fisher and Scott (1) were able to obtain a plastein by the action of pepsin on a concentrated peptic digest of insulin. This product was physiologically inert, and could not be crystallized. They suggested that trypsin, because of its presence in the pancreas, might yield more interesting results. This suggestion has been applied to the experiments reported here. It was necessary to use a peptic digest of insulin as a substrate for trypsin, since no one has yet been able to obtain a plastein from a tryptic digest.

We have also synthesized plasteins by the use of unactivated papain. The similarity of this enzyme to the intracellular proteinases seemed to offer a better opportunity for the production of a physiologically active plastein than the use of pepsin or trypsin. A papain digest of insulin and, in one trial, a pepsin digest of insulin were used as substrates for papain in the synthesis of the plasteins. No activator was used with the papain because of the danger of destruction of blood sugar lowering activity.

## EXPERIMENTAL

The procedure used for the hydrolysis of the protein and subsequent synthesis of the plastein was adapted from the method described by Fisher and Scott (1) for peptic hydrolysis and synthesis. These authors started with small quantities of protein. This procedure was modified in the case of tryptic synthesis to fulfil the optimum conditions described by Wasteneys and Borsook (2), and in the case of papain hydrolysis and synthesis to fulfil the optimum conditions reported by Collier (3).

When peptic digests were used, no attempt was made to remove the undigested protein before concentration and synthesis were begun, since it was present only in negligible quantities. In the case of papain digests, however, much undigested protein remained. Since this would interfere with the biological assay and the synthesis of plastein, we decided to remove this fraction before proceeding with the synthesis. This was done by heat coagulation. Du Vigneaud *et al.* (4) have shown that insulin dissolved in a 0.01 N HCl solution containing NaCl is completely precipitated by heating for 5 to 10 minutes. We found that insulin was precipitated by heating as follows: 10 mg. of insulin were placed in 10 cc.

of solution, the pH adjusted to 5.0, enough NaCl added to make a 0.17 M solution, and the solution heated in a boiling water bath for 1 hour. It was found in two trials that 94.7 and 97.3 per cent of the protein was precipitated.

A leveling bulb (without a side tube) which had been sealed off at the bottom was used to concentrate the solution. Graduations of 0.25, 0.5, and 1.0 cc. were marked on the tip. The concentration of the material was carried out with a pressure of 40 to 50 mm. of Hg, produced by means of a water-pump, and a temperature of 45–55°. Caprylic alcohol was added to prevent foaming.

The pH adjustments were made with 0.1 N HCl and 0.1 N NaOH; the pH determinations were made colorimetrically. Chloroform was used as a preservative in all experiments.

Nitrogen determinations were made by means of a modification of Pregl's micro-Kjeldahl method (5). Duplicates checked within 3 per cent. For the non-protein nitrogen determinations 10 per cent trichloroacetic acid was added to the samples to a concentration of 3.3 per cent, which has been shown by Charles and Scott (6) to be necessary for complete precipitation of insulin. The samples were allowed to stand at room temperature for 1 hour and then centrifuged. In all calculations the nitrogen of the enzymes is considered to be in the non-protein nitrogen fraction, since 3.3 per cent trichloroacetic acid does not precipitate them.

It was found inaccurate in these experiments to calculate directly the per cent of plastein synthesized. This was because of the small amount of material used and the loss during the process due to clinging of the material to the walls of the container. This material dried on the walls and could not be recovered. The following procedure was therefore used.

In those experiments in which the original protein was not precipitated after hydrolysis the ratio of non-protein to total nitrogen was determined after hydrolysis, this, when multiplied by 100, giving the per cent hydrolysis. After synthesis the total and non-protein nitrogen was again determined. The total nitrogen value was then expressed in terms of that which had existed as non-protein nitrogen before synthesis was begun. To obtain this corrected value it was assumed that the protein and the hydrolysis products were lost during synthesis in equal proportions, as found by Fisher and Scott (1). Thus, the total nitrogen after synthesis, when multiplied by that fraction of the total nitrogen before synthesis which was non-protein, gives the corrected value. In order to obtain the amount of plastein nitrogen present, the non-protein nitrogen after synthesis was subtracted from the total nitrogen after synthesis (corrected). The per cent synthesis was calculated as the ratio of plastein nitrogen to the total nitrogen after synthesis (corrected) multiplied by 100.



In those experiments in which the original protein was removed before synthesis by precipitation, the same method was used, except that it was not necessary to correct the value for total nitrogen after synthesis.

The effect on the blood sugar level of rabbits which had been fasted for 18 hours was used to test the physiological activity of the solutions. Two rabbits were used for each solution to be tested. The rabbits weighed between 2100 and 2900 gm. After a preliminary blood sample was withdrawn from an ear vein, an appropriate amount of the material was injected subcutaneously. Blood samples were then taken  $1\frac{1}{2}$ , 3, and 5 hours after the injection. In some cases samples were taken only  $1\frac{1}{2}$  and 4 hours after injection. The reducing sugar was determined by the method of Shaffer, Hartmann, and Somogyi (7, 8).

When trypsin was present, a part of the solution was treated in a manner described by Scott (9) as capable of dissociating any physiologically inactive insulin-trypsin complex present. This consisted of adding 10 cc. of a saturated alcoholic solution of benzoic acid and 5 cc. of 0.1 N HCl to 10 cc. of the plastin solution and letting it stand for 15 minutes. The alcohol was then nearly all driven off in a water bath, and the benzoic acid removed with ether. The solution thus obtained, after neutralization, was used for injection.

The following products were used in this work: Lilly's zinc insulin crystals; Difco 1:10,000 pepsin; Difco 1:100 trypsin; and Eimer and Amend's Mexican papain (1:200).

*Synthesis from Peptic Digests with Trypsin*—Two experiments were carried out. In each experiment 100 mg. of insulin were dissolved in 0.01 N HCl, and 25 mg. of pepsin added. The pH was adjusted to 1.9, and the solution, with a final volume of 52 cc., was incubated at 37° for 24 hours. Samples were then taken for nitrogen determinations and for biological tests. Two rabbits were used in each experiment, 2 cc. of digest being injected into each. The activity had been completely destroyed. The remaining 36 cc. were then adjusted to pH 5.7 and concentrated to 0.5 cc., after which 0.25 cc. of a solution containing 25 mg. of trypsin was added. The solution was then incubated at 37° for 4 days in Experiment I and 7 days in Experiment II. Distilled water was then added to a volume of 50 cc., and nitrogen determinations and biological tests performed. Four rabbits were used in each experiment, 2 cc. of untreated digest being injected into each of two animals, and 2 cc. of digest treated with benzoic acid, alcohol, and HCl into each of the other two.

*Synthesis from Papain Digests with Papain*—Three experiments were carried out. In Experiment III, 60 mg. of insulin were used, while in Experiments IV and V, 80 mg. were used. In each experiment the insulin was placed in solution with 25 mg. of papain, and the pH adjusted to 4.7. The solution, with a total volume of 30 cc., was incubated for 3 days at



# THE DEVELOPMENT OF CYTOCHROME OXIDASE IN THE CHICK EMBRYO\*

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Studies on the development of enzyme systems with time in animal embryos are relatively rare. Especially is this true of the chick embryo, of which the only complete study of enzyme development appears in the work of Levy and Palmer (1) on dipeptidase accumulation. According to these authors, dipeptidase activity in the chick increases with time and this increase is correlated with the accumulation of total nitrogen which presumably comes from the yolk. The same kind of correlation between nitrogen increase and enzyme activity has been demonstrated in oat seedlings for catalase and dehydrogenase activity by Albaum, Donnelly, and Korkes (2).

In the chick, cytochrome *c* does not appear until about the 4th day, according to Yaoi (3), and Potter and DuBois (4) were unable to detect it until about the 6th day. The present work was undertaken to ascertain when the enzyme, cytochrome oxidase, which usually operates with cytochrome *c* as part of the same system, appears and how its concentration changes during the course of early development.

## *Material and Methods*

Chick eggs were incubated at 37-38° for lengths of time varying from 2 to 12 days. The embryos were removed from the yolk and dissected away from the extraembryonic membranes. Extracts of the embryos were prepared in the following manner. One to eight embryos was used, depending on the age. These were ground in a small quantity of sand and taken up in 2 cc. of *m*/15 phosphate buffer ( $\text{Na}_2\text{HPO}_4$ ,  $\text{KH}_2\text{PO}_4$ ) of pH 7.4. After light centrifugation, the supernatant fluid was decanted and used immediately for enzyme assay.

Cytochrome oxidase activity was determined by measuring the oxygen consumption of the extracts in the presence of *p*-phenylenediamine (0.433 gm. per 100 cc.) and cytochrome *c* in a Warburg respirometer at 26°. The usual procedure was to use 0.2 cc. of extract, 1.0 cc. of *p*-phenylenediamine, 0.4 cc. of cytochrome *c*, and 0.4 cc. of *m*/15 phosphate buffer.

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Contribution from the Department of Biology, Brooklyn College, No. 41.

Control runs were carried out in the absence of cytochrome *c* and *p*-phenylenediamine. The cytochrome *c* was prepared from beef heart by the method of Keilin and Hartree (5). The presence of cytochrome oxidase was tested for, in addition, by treating with 0.001 *M* sodium azide which, according to Keilin (6), specifically inhibits the oxidase. Measurements of total nitrogen were made on extracts by means of a micro-Kjeldahl technique in the usual way.

#### EXPERIMENTAL

The total oxygen consumption in the presence of *p*-phenylenediamine and cytochrome *c*, together with the total nitrogen content of the extracts

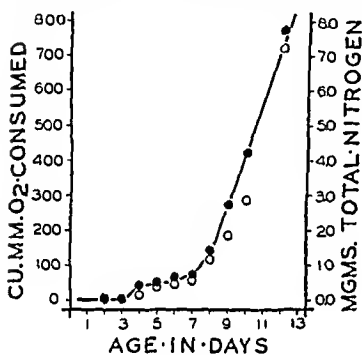


FIG. 1

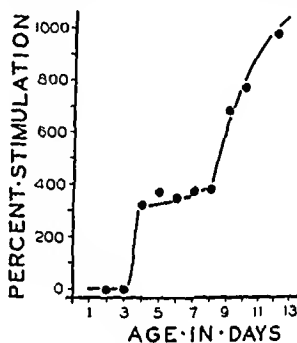


FIG. 2

FIG. 1. Relationship of rate of oxygen consumption (c.mm. of  $O_2$  consumed per hour per embryo) in the presence of *p*-phenylenediamine and cytochrome *c* (closed circles), total Kjeldahl nitrogen in extract from one embryo (open circles), and time of incubation.

FIG. 2. Relationship between percent stimulation of oxygen uptake in the presence of *p*-phenylenediamine and cytochrome *c* over control respiration in the absence of both, with time.

on a per embryo basis, is shown in Fig. 1. It is apparent that, as in the work of Levy and Palmer (1) on dipeptidase activity, there is a correlation between nitrogen accumulation and cytochrome oxidase activity. Both curves show a break at about 4 days and a sharp increase in nitrogen content and enzyme activity at about 8 days. Levy and Palmer find a break at about  $4\frac{1}{2}$  days and another at about  $10\frac{1}{2}$  days for dipeptidase.

The earlier break in the curve for cytochrome oxidase is of especial interest. Its significance becomes much clearer when the oxidase data are replotted in terms of per cent stimulation over the oxygen uptake of the control, as in Fig. 2. Plotting the data in this manner shows that

there is no stimulation in uptake until the 4th day. This can only mean that the enzyme does not appear until that time. That this interpretation is correct is indicated by the effect of sodium azide, shown in Table I. Sodium azide does not begin to inhibit oxygen uptake until the 4th day. The absence of enzyme until the 4th day fits in with the observation reported by Yaoi (3) that cytochrome is not present until this same time.

The data plotted in Fig. 2 also show the sharp break in enzyme activity at the 8th day. At this time, the nitrogen content and enzyme activity increase sharply. This may be related to the change in the rate and character of the metabolism of the embryo which occurs at about that time. All of the pertinent data are presented by Needham (7). Up until about the 9th day, carbohydrate is the chief source of energy and the metabolic

TABLE I

*Inhibition of Oxygen Uptake in Presence of p-Phenylenediamine and Cytochrome c by 0.001 M NaN<sub>3</sub>*

Age	Inhibition	Age	Inhibition
<i>days</i>	<i>per cent</i>	<i>days</i>	<i>per cent</i>
2	0	7	73
3	0	8	64
4	45	9	62
5	66	11	76
6	59	12	69

rate is low. On about the 7th day, fat begins to be utilized and continues to be burned at an increasingly rapid rate up until the time of hatching.

## SUMMARY

1. The change in cytochrome oxidase activity in the chick embryo has been measured from 2 to 12 days of incubation.

2. Cytochrome oxidase increase is correlated with increase in total nitrogen.

3. Cytochrome oxidase, as measured by oxygen uptake in the presence of cytochrome *c* and *p*-phenylenediamine does not appear until the 4th day of incubation. Neither can the oxygen uptake of extracts with or without *p*-phenylenediamine and cytochrome *c* be inhibited with sodium azide until the 4th day of incubation.

4. There is a sharp increase in enzyme activity at the 8th day of incubation. This increase is briefly discussed in terms of the change in the rate and character of the metabolism known to take place at about that time.

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# MODIFIED METHODS FOR THE CHEMICAL AND BIOLOGICAL DETERMINATION OF CHOLINE\*

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Recent investigations, which have demonstrated the importance of choline as a dietary constituent for the rat, have created considerable interest regarding the total choline content of foods and other biological materials. Fletcher and coworkers (1) determined the total choline content of a number of substances by assaying the acetylated choline on the isolated intestine of the rabbit. These workers stated that duplicate assays occasionally differed by almost 30 per cent. Jacobi and coworkers (2), using a method based on the precipitation of choline as the reineckate (3), and comparing this procedure with a biological assay procedure (4), found satisfactory agreement between these two methods. Jacobi and coworkers also reported that values for the choline content of tissues, as determined by the reineckate method, agreed satisfactorily with the results obtained by Fletcher and coworkers (1).

The present paper is concerned with the details of a modified chemical method for the determination of total choline in foods and other biological materials and the development of a biological assay procedure for the determination of the protective value of such materials when fed to rats receiving a choline-deficient diet. Data are presented to show that the results obtained by the chemical procedure are consistently higher than those reported previously by other investigators. Furthermore, the results indicate that the rat assay will prove valuable in determining the total protective value of foods and other biological materials; this value includes not only choline but other substances behaving like choline in the prevention of the hemorrhagic disease due to choline deficiency.

## Methods

*Chemical Determination*—A sample of biological material was chosen which contained from 4 to 8 mg. of choline. The sample was weighed and transferred to a fiber extraction thimble. The thimble was placed in the bottom of a Bailey-Walker extraction cylinder containing 30 ml. of absolute methanol. After 4 hours extraction at the boiling temperature

\* Approved for publication by the Director of the Alabama Agricultural Experiment Station. A preliminary report of this work was presented before the annual meeting of the American Institute of Nutrition at Boston, April 1, 1942.

of the solvent, the thimble containing the sample was raised, permitted to drain, and the solvent transferred to a 125 ml. filtering flask. The thimble was then returned to the extraction cylinder, 30 ml. of fresh solvent added, and the extraction continued for about 16 hours. The solvent was again removed and the sample extracted a third time for 4 hours with 30 ml. of fresh solvent.

The combined extracts were reduced to near dryness in a water bath (60–70°) under reduced pressure. The residue was treated with 30 ml. of saturated aqueous barium hydroxide for 2 hours at 100°. After being allowed to cool, the hydrolysate was neutralized to phenolphthalein with acetic acid and filtered by suction through an asbestos pad into another 125 ml. filtering flask. The flask used in hydrolysis and the asbestos pad were washed with six 2 ml. portions of distilled water. 6 ml. of a 2 per cent solution of Reinecke salt in methanol were added to the combined filtrate and washings and the flask was placed in a refrigerator (3°) for 4 hours to allow complete precipitation.

The precipitate of choline reineckate was filtered onto an asbestos pad. The flask and precipitate were washed three times with 2.5 ml. portions of cold ethanol (3°). Air was drawn through the pad until the precipitate was thoroughly dry. The precipitate was dissolved by washing with acetone, filtered, and made to 25 ml. volume. The concentration of choline reineckate was determined by means of a photoelectric colorimeter,<sup>1</sup> Filter 520 being used.

For the calibration of the instrument, twelve solutions containing varying concentrations of pure choline chloride were prepared. Each of these solutions was carried through the procedure for choline determination as just outlined. From these data, a standard curve was made, *L* values being plotted against mg. of choline chloride per 10 ml. of acetone solution.

*Biological Assay*—The biological assay consisted of comparing the amount of protection afforded by a food material and by pure choline chloride in preventing kidney hemorrhages which occurred in rats receiving a choline-deficient diet. A diet developed in this laboratory (5), which was severely deficient in choline and in other substances behaving like choline (Diet 31-PMC), was used as the basal diet in these experiments.

Previous experiments (6) had indicated that rats from different litters, receiving this diet plus suboptimum levels of choline, varied considerably in their susceptibility to choline deficiency. For this reason, litter mate weanling rats, weighing 40 to 45 gm., were divided into two like groups as to number, weight, and sex. One group received the choline-deficient diet plus that amount of the test food material daily which would supply

<sup>1</sup>The photoelectric colorimeter used was of the Evelyn type, manufactured by the Rubicon Company of Philadelphia.



4 mg. of choline chloride as determined by the chemical procedure; the other group received the same basal diet plus 4 mg. of pure choline chloride. Smaller and larger amounts of the test material were also fed and compared with the 4 mg. level of pure choline chloride. Several litters of rats were used in testing any given material, as is indicated in the condensed results in Tables VII and VIII.

The 4 mg. level of choline chloride was chosen because at this level of daily intake some rats developed kidney hemorrhage, while others did not (6). The number of animals affected in any particular feeding test was thus used as a criterion in determining the relative protective value of the test material and pure choline chloride.

## RESULTS AND DISCUSSION

### *Chemical*

*Extraction of Choline*—Methanol was used as the solvent for extracting the choline from the food material, because higher values were obtained with it than with any of the other solvents tried. Data presented in Table I show the effectiveness of a number of solvents. In general, the alcohols were the most effective, the highest values being obtained with methanol. Assuming that methanol removed 100 per cent, chloroform-methanol removed about 80 per cent and alcohol-ether removed about 34 per cent of the choline from yeast. Alcohol-ether removed about 50 per cent of the choline from dried beef or pork liver and only 20 per cent from peanut meal. This solvent was considerably more effective on a sample of fresh rat liver, from which it removed 80 per cent of the choline. Although solvents varied in their effectiveness, depending upon the material being extracted, for all the materials used, methanol was superior to any other solvent for extracting choline.

*Alkaline Hydrolysis*—The alkaline hydrolysis with aqueous barium hydroxide was conducted at the boiling temperature because of the failure to obtain consistent results at 80°, the temperature suggested by Jacobi and coworkers (2). As is seen in Table II, hydrolysis at the boiling temperature for 2 hours produced higher and more uniform results than were obtained at 80°. That choline chloride was not destroyed by a 2 hour treatment at the boiling temperature is also shown in Table II.

*Choline Recovery*—To determine whether substances present in the methanol extracts might interfere with the determination of choline, known quantities of pure choline chloride were added to extracts of peanut meal, yeast, and liver (Table III). Excellent recoveries were obtained in all cases, the maximum deviation from theoretical values being 1.5 per cent.

*Stability of Choline to Heat Treatment*—To determine the stability of choline to heat treatments generally employed for the removal of moisture

from biological materials, analyses were conducted on samples of fresh liver as well as on samples of the same liver dried at 104° for 24 and 48 hours. The data presented in Table IV indicate that choline is stable to the heat treatment used for routine moisture determinations.

*Other Possible Reineckates*—To determine whether methanol extracts of tissues such as yeast, liver, or peanut meal might contain substances which would be precipitated by Reinecke salt along with choline, a number

TABLE I

*Efficacy of Solvents for Extraction of Choline from Biological Material*

The dried materials were ground to pass a 40 mesh screen. The fresh material was ground to a pulp with sea sand before being extracted. The extractions were conducted for 24 hours at the boiling temperature of the solvent; three 30 ml. quantities of solvent were used on each sample.

Material	Solvent	Choline chloride extracted	
		mg. per gm.	per cent
Dried brewers' yeast	Methanol	4.97	100.0
	Methanol-ethanol (1:1)	4.79	96.3
	Ethanol	4.75	95.8
	Methanol-chloroform (1:1)	3.96	79.7
	Ethanol-Skellysolve B (1:1)	2.42	48.7
	Ethanol-ethyl ether (1:1)	1.68	33.8
	Chloroform	1.65	33.2
	Acetone	0.84	16.9
	Ethyl ether	0.60	12.1
	Skellysolve B	0.33	6.6
Dried beef liver	Petroleum ether	0.15	3.1
	Methanol	23.80	100.0
	Ethanol-ethyl ether (1:1)	13.50	56.7
" pork "	Methanol	21.85	100.0
	Ethanol-ethyl ether (1:1)	11.38	52.0
Peanut meal	Methanol	2.28	100.0
	Ethanol-ethyl ether (1:1)	0.49	21.2
Fresh rat liver	Methanol	3.55	100.0
	Ethanol-ethyl ether (1:1)	2.83	80.0

of substances were tested. Thiamine and nicotinic acid, in slightly acidified aqueous solutions, formed pink, acetone-soluble precipitates with Reinecke salt. If these compounds were treated for 2 hours with boiling aqueous barium hydroxide, however, they failed to produce any precipitate. Negative results were also obtained, both before and after alkaline treatment, with samples of pyridoxine, riboflavin, calcium pantothenate, ascorbic acid, inositol, betaine, methionine, creatine, ethanolamine, ammonia, urea, and *p*-aminobenzoic acid.

*Solubility of Choline Reineckate in Ethanol*—In calibrating the photo-electric colorimeter, as previously stated, the choline reineckate used in

TABLE II

*Effect of Temperature upon Liberation of Choline from Methanol Extracts with Aqueous Barium Hydroxide*

Material	Treatment for 2 hrs.	Choline chloride found	
			Average
	°C.	mg. per gm.	mg. per gm.
Extract of beef liver	80	18.84	20.47
		20.10	
		22.46	
	100	24.42	24.49
		24.46	
Extract of dried brewers' yeast	80	24.60	24.49
		4.67	
		4.89	
	100	4.50	4.69
		4.95	
6.53 mg. choline chloride	100	4.98	4.98
		5.00	
		6.60	
		6.52	
		6.52	6.55

TABLE III

*Recovery of Choline Chloride Added to Methanol Extracts of Yeast, Liver, and Peanut Meal*

Methanol extract of	Choline chloride				
	In extract	Added	Total present	Total found	
	mg.	mg.	mg.	mg.	per cent
Dried brewers' yeast	3.40	1.69	5.09	5.05	99.2
" beef liver	1.88	2.11	3.99	3.95	99.0
	1.88	3.81	5.69	5.70	100.2
	2.18	2.11	4.29	4.35	101.5
	2.64	2.96	5.60	5.63	100.5
	3.77	2.11	5.88	5.90	100.2
	4.52	2.11	6.63	6.60	99.5
	1.45	3.38	4.83	4.83	100.0
Peanut meal	2.90	1.69	4.59	4.58	99.8
	2.90	1.69	4.59	4.62	100.6

obtaining a standard curve was uniformly washed with small quantities (three 2.5 ml. portions) of cold ethanol before dissolving in acetone. This

was highly important, since excessive washing with ethanol resulted in appreciable losses of choline, as is shown in Table V. When the *L* values obtained with choline reineckate which had been washed three times with 2.5 ml. portions of cold ethanol are compared with the *L* values obtained from choline reineckate which had been washed six times with 5 ml.

TABLE IV  
*Effect of Heat Treatment on Stability of Choline in Rat Liver*

Liver from Rat No.	Treatment	Choline chloride found*
		<i>mg. per gm.</i>
1	None	3.55
1	Dried at 104° for 24 hrs.	3.52
2	None	3.79
2	Dried at 104° for 48 hrs.	3.82

\* All the results were calculated on the fresh basis.

TABLE V  
*Choline Reineckate Concentration and Corresponding L Values*

Choline chloride in 10 ml. acetone	<i>L</i> value* (2 - log <i>G</i> )	<i>L</i> value† (2 - log <i>G</i> )
<i>mg.</i>		
0.631	0.0706	0.0206
1.263	0.1503	0.0410
1.894	0.2236	0.0928
2.525	0.3140	0.1565
3.157	0.3980	0.2347
3.788	0.4910	0.3140
4.420	0.5770	0.3900
5.051	0.6580	0.4690
5.682	0.7450	0.5450
6.314	0.8380	0.6240
6.945	0.9030	0.7040
7.576	0.9890	0.7760

\* The *L* values which were obtained with choline reineckate which had been washed three times with 2.5 ml. portions of cold ethanol.

† The *L* values which were obtained when the choline reineckate was washed six times with 5 ml. portions of cold ethanol.

portions, it is seen that considerable loss of choline had occurred with the more intensive washing. To determine the solubility of choline reineckate in ethanol a known quantity of the crystalline substance was shaken with ethanol and allowed to stand at room temperature for 16 hours. By this treatment choline reineckate was soluble to the extent of 19 mg. per 100 ml. In routine determinations, therefore, each sample of choline reineckate

was washed three times with 2.5 ml. of cold ethanol, as was done in calibrating the colorimeter.

The standard curve was checked from time to time with standard solutions of choline reineckate which were prepared according to the procedure outlined. Between *L* values of 0.2236 and 0.7450 the maximum error encountered was  $\pm 1.5$  per cent; this error increased to  $\pm 5$  per cent for *L* values from 0.0706 to 0.2236 and from 0.7450 to 0.9890.

TABLE VI  
*Total Choline Content of Biological Material*

Material	Choline chloride	Material	Choline chloride
	<i>mg. per gm.</i>		<i>mg. per gm.</i>
Dried brewers' yeast, Sample 1	5.05	Soy bean meal	3.40
	4.98		3.50
	4.95		3.45
" " " " 2	4.90	Mature soy beans	3.45
	4.89		3.40
	4.90		3.35
" " " " 3	3.03	Dried beef liver	23.80
	3.00		23.95
	3.07		23.60
" bakers' " " 1	5.25	" pork "	21.94
	5.15		22.06
	5.19		21.56
" " " " 2	4.45	" rat " Sample 1*	11.71
	4.45		11.42
	4.48		11.71
" " " " 3	4.13	" " " " 2*	10.26
	4.20		10.19
	4.24		10.14
Peanut meal	2.22	" " " " 3*	3.76
	2.25		3.83
	2.28		3.80

\* Sample 1 was obtained from a rat on the stock diet. Sample 2 was from a rat receiving a choline-deficient diet plus 20 mg. of choline chloride daily for 6 weeks. Sample 3 was from a rat receiving the choline-deficient diet only for 6 weeks.

*Analyses for Total Choline*—Choline determinations were made on a number of substances by the procedure as previously outlined. Results of triplicate analyses on each material are presented in Table VI. Different strains of yeast varied considerably in their choline content. Also differences were noted in the choline content of liver tissue from different mammalian species. Extremely low choline values were obtained for liver tissue from rats which had received a choline-deficient diet for 6

weeks. A sample of soy bean meal contained as much choline as did whole mature soy beans. In general, the values reported here for yeast, beef liver, pork liver, and normal rat liver are 30 to 50 per cent higher than the values reported by other workers (1, 2).

### Biological

Bioassay results with peanut meal are presented in Table VII. When peanut meal was fed at a level to supply 4 mg. of choline chloride daily, as determined by the reineckate procedure, the same protection was exhibited as with pure choline chloride fed at the 4 mg. level. In each group, ten out of twelve rats had either died with kidney hemorrhage, or exhibited hemorrhage or signs of former hemorrhage on the 14th day of

TABLE VII

*Comparison of Protective Value of Peanut Meal and Pure Choline Chloride When Fed to Rats Receiving Choline-Deficient Diet*

No. of rats	Daily supplement	No of rats with kidney hemorrhage
12	1 760 gm. peanut meal ( $\approx$ 4 0 mg. choline chloride)	10
12	4 0 mg. choline chloride	10
16	Diet 41-P* ( <i>ad libitum</i> )	11
16	" 31-PMC + choline† ( <i>ad libitum</i> )	11
11	1 580 gm peanut meal ( $\approx$ 3 6 mg choline chloride)	10
11	4 0 mg choline chloride	7

\* Diet 41-P was the same as Diet 31-PMC except that the alcohol extracted peanut meal was replaced by crude unextracted peanut meal

† Choline chloride was added to restore the choline content of the diet to that of Diet 41-P

the experiment when necropsy examinations were routinely made. Similar results were obtained when the rats received a basal diet containing crude unextracted peanut meal, or a diet containing the extracted meal with its original choline content restored by the addition of pure choline chloride. In each group, eleven out of sixteen rats had either died or showed kidney hemorrhage at necropsy. When the choline-deficient diet was supplemented with crude peanut meal at a level to supply 3.6 mg. of choline chloride, the incidence of kidney hemorrhage was greater than if 4 mg. of choline chloride were fed. These results showed definitely that for peanut meal the reineckate procedure for choline determination was in good agreement with the bioassay results.

Bioassays were also conducted on samples of dried brewers' yeast and dried beef liver which had been analyzed for choline by the reineckate

procedure. The results are presented in Table VIII. The same protection was observed with yeast fed at a level to supply 4 mg. of choline chloride as was observed when the same level of pure choline chloride was fed. Feeding 10 per cent more or 10 per cent less yeast resulted in slightly greater or slightly less protection against kidney hemorrhage.

Dried beef liver gave more protection than could be accounted for on the basis of its choline content as determined chemically. Liver fed at a level to supply 3.2 mg. of choline chloride gave the same protection as 4 mg. of pure choline chloride. Smaller or larger amounts of liver, when compared to 4 mg. of choline chloride, gave correspondingly less or greater

TABLE VIII

*Comparison of Protective Value of Yeast or Liver and Pure Choline Chloride When Fed to Rats Receiving Choline-Deficient Diet*

No of rats		No of rats with kidney hemorrhage
11	0.778 gm yeast ( $\approx$ 4.0 mg choline chloride)	3
11	4.0 mg choline chloride	3
11	0.700 gm yeast ( $\approx$ 3.6 mg choline chloride)	6
11	4.0 mg choline chloride	4
8	0.856 gm yeast ( $\approx$ 4.4 mg choline chloride)	4
8	4.0 mg choline chloride	6
12	0.130 gm liver ( $\approx$ 3.2 mg choline chloride)	6
12	4.0 mg choline chloride	6
4	0.114 gm liver ( $\approx$ 2.8 mg choline chloride)	2
4	4.0 mg choline chloride	0
7	0.146 gm liver ( $\approx$ 3.6 mg choline chloride)	2
7	4.0 mg choline chloride	4
8	0.163 gm liver ( $\approx$ 4.0 mg choline chloride)	4
8	4.0 mg choline chloride	8
4	0.179 gm liver ( $\approx$ 4.4 mg choline chloride)	1
4	4.0 mg choline chloride	4

protection. These results indicate that liver contained substances, other than choline, which exhibited a choline-like action.

The bioassay should prove valuable as a means of determining the combined effect of choline, and other substances present in biological material which behave like choline, in the prevention of kidney hemorrhage in the rat. Use of the reineckate precipitation procedure, along with the bioassay, would yield information concerning the relationship between the choline content of various materials and their biological protective value.

It is apparent, from the results presented in Tables VII and VIII, that there was considerable litter variation in the susceptibility of rats

to choline deficiency. The uniformity of the results obtained, however, when bioassay comparisons were restricted to rats within a litter, indicated that this difficulty could be largely overcome. Furthermore, the results indicate that the bioassay procedure is a highly sensitive biological test, since variations of 10 per cent in the test food material could be detected.

#### SUMMARY

The reineckate method for the determination of total choline in foods and other biological materials has been improved. A biological assay has been developed for determining the total choline value of such materials. A combination of chemical determination and biological assay should prove valuable in arriving at both the choline content of biological material and its content of substances other than choline which exhibit choline-like action when fed to rats.

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# INHIBITION OF CATALASE BY HYDROXYLAMINE AND *p*-HYDROXYLAMINO BENZENESULFONAMIDE AND THE REVERSAL OF INHIBITION BY SERUM, CRYSTALLINE SERUM ALBUMIN, AND HEMIN\*

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In a previous communication (1) it was shown that *p*-hydroxylaminobenzenesulfonamide (HONHR) did not inhibit catalase in the presence of serum. In contrast in a later experiment it was observed that HONHR inhibited catalase in the absence of serum. The present study deals with the reversible inhibition of catalase by hydroxylamine and its benzenesulfonamide derivative in the presence of serum, crystalline serum albumin, and hemin.

*p*-Hydroxylaminobenzenesulfonamide was obtained in two forms (1), one melting at 141.5° and the other at 161.5°. These two forms manifest different chemical and physical properties. A paper dealing with these differences will be published elsewhere. The experiments in this study were carried out with the substance which melts at 141.5°. It oxidizes with relative ease; a study of its biological and chemical action therefore involves certain complications which must be strictly controlled to measure its effects accurately. 1 mole of this substance consumes 1 mole of oxygen, yielding 1 mole of *p*-nitrosobenzenesulfonamide and 1 mole of hydrogen peroxide. The former reaction product combines with 1 mole of *p*-hydroxylaminobenzenesulfonamide forming azoxydibenzenesulfonamide. The rate of oxidation and, therefore, the amount of hydrogen peroxide formed are dependent on the pH of the reaction system. At pH 4.5 it is practically non-oxidizable; on the other hand in alkaline solution the volume of oxygen consumed corresponds to 90 to 95 per cent of the theoretical value. At neutrality the degree of oxidation occupies an intermediary point.

In the inhibition experiments if catalase is present in excess, hydrogen peroxide which is produced by the oxidation of *p*-hydroxylaminobenzenesulfonamide (inhibitor) is not to be found in the reaction system. Even though the catalase may be completely inhibited, the oxidation of the inhibitor is not inhibited. This inhibitor thus provides the catalase it in-

\* This work was carried out under grants from the Josiah Macy, Jr., Foundation and Merck and Company.

hibits with the specific substrate and thereby offers an excellent method of studying its effect on catalase under various conditions (Table I). Addition of hydrogen peroxide to the system does not affect the inhibition results. These methods have been employed in this study in the presence and absence of serum, crystalline serum albumin, and hemin, on blood and crystalline liver catalases (Tables II and III).

#### EXPERIMENTAL

*Crystalline Liver Catalase*—The sample of crystalline liver catalase kindly supplied by Professor J. B. Sumner of Cornell University contained 23.4 mg. of non-dialyzable total solids per cc. of stock solution. In inhibition experiments the solutions of catalase used were prepared by diluting the undialyzed stock sample. The expressed molarity was calculated from the molecular weight of 248,000 as determined by Sumner and Gralén (2). Since 1 molecule of catalase contains four heme groups, a molar solution of catalase would be equal to 4 M with respect to heme.

*Crystalline Serum Albumin*—The dry weight of a dialyzed solution of crystalline serum albumin was determined by analysis for micro-Kjeldahl nitrogen. The molecular weight of 70,000 was used to calculate the molarity of albumin per vessel. The absence of hemin in serum albumin was shown with benzidine as follows: 1.63 mg. of serum albumin in 0.5 cc. of phthalate buffer of pH 4.4 were treated with 3 drops of 3 per cent  $\text{H}_2\text{O}_2$  solution and 2 drops of saturated alcoholic benzidine solution. The test was negative. As control, 0.5 cc. of a solution containing  $3.6 \times 10^{-5}$  mg. of hemin ( $1.1 \times 10^{-7}$  M) gave a positive peroxidative test.

*Hemin*—The hemin (mol. wt. 651.3) used in this study was a crystalline preparation kindly supplied by Dr. D. L. Drabkin of the Department of Physiological Chemistry.

*Blood Catalase*—The solution of blood (rabbit) catalase was water-clear. In the preparation of the stock solution care was taken to remove the last traces of serum and hemoglobin as previously described (3). 1 cc. of catalase solution was obtained from 0.1 cc. of the rabbit whole blood.

*Normal Serum*—The normal horse serum was heated at  $56^\circ$  (water bath) for 1 hour to inactivate traces of catalase that might have been present. Though it was free from apparent traces of hemolyzed blood, it gave a positive peroxidative test with benzidine, showing the presence of hematin derivatives. Dialysis of the serum against running 0.02 N HCl solution and distilled water for several days did not eliminate the substance responsible for the positive peroxidative test.

*Measurement of Inhibition of Catalase*—The inhibition of catalase was measured manometrically with the Barcroft-Warburg set-up, and at the end of the experiment the contents of the vessels were also analyzed for  $\text{H}_2\text{O}_2$  (iodometric method).

The manometric measurements were carried out in vessels with two side arms. The reaction system consisted of 2.5 cc. of  $M/15$  phosphate buffer of pH 7.4, 0.2 cc. of catalase solution, and 1.0 cc. of either serum, serum albumin, or hemin solution. One side arm contained the inhibitor, the other side arm the  $H_2O_2$  (0.0063  $M$  final concentration). Distilled water was added to the vessel to bring the final volume to 5.8 cc. After the equilibration of the temperature the contents of the side arms were mixed in the order desired.

At the end of the manometric measurements the contents of the vessels and the side arms were thoroughly washed into Erlenmeyer flasks to which were added 2.0 cc. of 5 per cent  $H_2SO_4$  solution, 2.0 cc. of 2 per cent potassium iodide solution, and 1.0 cc. of 1 per cent ammonium molybdate solution (containing 5 cc. of 25 per cent  $H_2SO_4$  solution). After a 5 minute interval the liberated iodine was determined by titrating with 0.01  $N$  sodium thiosulfate solution delivered by a micro burette. Near the end-point 2 drops of 1 per cent soluble starch solution were used.

#### RESULTS AND DISCUSSION

The results given in Table I show that *p*-hydroxylaminobenzenesulfonamide (HONHR) inhibited rabbit blood and liver catalase.<sup>1</sup> The inhibition caused by  $9.17 \times 10^{-3} M$  HONHR was completely reversed by 1.0 (or 0.01) cc. of clear catalase-free serum.<sup>2</sup> In other experiments similar to those presented in Table I,  $1 \times 10^{-4} M$  HONH<sub>2</sub> inhibited  $1.63 \times 10^{-9} M$  crystalline liver catalase 60.0 per cent, and  $2.72 \times 10^{-10} M$  liver catalase was inhibited 59 per cent by  $1 \times 10^{-5} M$  HONH<sub>2</sub>. These inhibitions were similarly reversed by serum.

It is evident from these data that the serum contains certain substances which compete with catalase for the above inhibitors. In peroxidative tests serum showed the presence of a hematin compound which could not be eliminated by dialysis. It was thought that either the serum proteins or the hemin, or both, present in serum might be responsible for the complete reversal of the inhibition of catalase by the above inhibitors. As will be seen from the results given in Tables II and III, this assumption was confirmed. Table II shows that  $2.72 \times 10^{-10} M$  crystalline liver catalase was inhibited 89 per cent by  $1 \times 10^{-5} M$  HONH<sub>2</sub> and 89 per cent by  $4.58 \times 10^{-5} M$  HONHR. These inhibitions were reversed to the extent of 77 and 96.0 per cent, respectively, by  $4.0 \times 10^{-6} M$  crystalline serum

<sup>1</sup> Collier (4) had previously shown that *p*-hydroxylaminobenzenesulfonamide inhibited liver catalase.

<sup>2</sup> The present study shows that in the presence of serum 10 mg. ( $9.17 \times 10^{-3} M$ ) of *p*-hydroxylaminobenzenesulfonamide are incapable of inhibiting 0.02 cc. of catalase (corresponding to 0.002 cc. of whole blood). In other words, 5 gm. of *p*-hydroxylaminobenzenesulfonamide are incapable of inhibiting 1.0 cc. of whole blood.

albumin. The inhibition by  $1 \times 10^{-5}$  M HONH<sub>2</sub> was reversed 46 per cent also with as little as  $6.2 \times 10^{-8}$  M crystalline serum albumin. This indicates that HONH<sub>2</sub> has about the same affinity for serum albumin that it has for catalase. The question as to what group in the protein reacts with the inhibitors to account for the reversal of the inhibition of catalase was investigated.

Carbohydrates such as mannose, galactose, levulose, arabinose, polysaccharides of pneumococcus Type I, and rabbit liver glycogen were added

TABLE I

*Reversibility of Inhibition of Rabbit Blood and Crystalline Liver Catalase by p-Hydroxylaminobenzenesulfonamide (HONHR) in Presence of Serum*

C.mm. of O<sub>2</sub> consumed by  $9.17 \times 10^{-3}$  M HONHR and the decomposition of the H<sub>2</sub>O<sub>2</sub> formed by catalase. Reaction period 150 minutes.

Reaction systems	O <sub>2</sub>	H <sub>2</sub> O <sub>2</sub>	Inhibition	Reversal
	c.mm.	micromoles*	per cent	per cent
1. No catalase.....	350	8.7		
2. " " + 1 cc. serum.....	459	11.1		
3. 0.02 cc. blood catalase†.....	354	8.6	99	
4. 0.02 " " + 1 cc. serum....	260	0.0	0	100
5. 0.2 " " ".....	253	1.25	14	
6. 0.2 " " " + 1 cc. serum....	269	0.0	0	100
7. $1.63 \times 10^{-9}$ M liver catalase.....	331	7.65	77	
8. $1.63 \times 10^{-9}$ " " " + 1 cc. serum.....	267	0.0	0	100
9. $3.62 \times 10^{-9}$ M liver catalase.....	310	3.15	36	
10. $3.62 \times 10^{-9}$ " " " + 1 cc. serum.....	264	0.0	0	100

In comparison to Systems 1, 2, 3, 7, and 9, Systems 4, 5, 6, 8, and 10 show smaller volumes of oxygen consumed. This is due to the fact that in the latter systems catalase is not inhibited and therefore it decomposes H<sub>2</sub>O<sub>2</sub> formed according to  $2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$ . The liberated oxygen returning to the system causes a positive pressure and therefore the measured volume of consumed O<sub>2</sub> is smaller. 47 to 60 per cent of the reactions were completed after 30 minutes in all the above systems. After 60 minutes the completed reactions varied from 60 to 90 per cent.

\* Cc. of 0.01 N Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>  $\times$  5.

† 0.02 cc. of blood catalase corresponds to 0.002 cc. of whole rabbit blood.

to the reaction systems. There was no trace of reversal of the inhibition of catalase, indicating either the absence of a carbohydrate prosthetic group in crystalline serum albumin (5) or the inability of the inhibitors to react with the protein-bound carbohydrates under these experimental conditions. To test the possible reactivity of the keto group in dipeptide linkages for the inhibitors hippuric acid was used, but no effect was observed. On the basis of these results it would seem that the protein molecule itself manifests affinity for HONH<sub>2</sub> and HONHR.

Table III showed that  $2.72 \times 10^{-10}$  M crystalline liver catalase was inhibited 84.0 per cent by  $1 \times 10^{-5}$  M HONH<sub>2</sub> and about 70 per cent by  $4.58 \times 10^{-5}$  M HONHR. The inhibition by HONH<sub>2</sub> was reversed 52 to 64.0 per cent by  $4.73 \times 10^{-5}$  M hemin. These results show that HONH<sub>2</sub>

TABLE II

*Reversibility of Inhibition of Crystalline Liver Catalase by Hydroxylamine and p-Hydroxylaminobenzenesulfonamide (HONHR) in Presence of  $4.0 \times 10^{-5}$  M Crystalline Serum Albumin*

Reaction period 150 minutes

Reaction systems	O <sub>2</sub>	H <sub>2</sub> O <sub>2</sub>	Inhibition		Reversal	
			Iodo metri- cally	Mano metri- cally	Iodo metri- cally	Mano metri- cally
2 72 × 10 <sup>-10</sup> M liver catalase						
	c mm	micro moles	per cent	per cent	per cent	per cent
1 No inhibitor	430	9 3				
2 " " + serum albumin	537	1 4				
3 1 × 10 <sup>-5</sup> M HONH <sub>2</sub>	76	41 5	89	83*		
4 1 × 10 <sup>-5</sup> " " + serum albumin	428	10 3	21	20†	77†	75†
5 4 58 × 10 <sup>-5</sup> M HONHR	64	41 6	89	85*		
6 4 58 × 10 <sup>-5</sup> " " + serum al- bumin	510	1 5	4	5†	96†	91†
Catalase absent Controls						
7. 1 × 10 <sup>-5</sup> M HONH <sub>2</sub>	15	46 7				
8 1 × 10 <sup>-5</sup> " " + serum albumin	0	49 5				
9 4 58 × 10 <sup>-5</sup> M HONHR	5	47 0				
10 4 58 × 10 <sup>-5</sup> " " + serum al- bumin	5	41 8				
11 Serum albumin, no inhibitor	2	44 9				

\* The inhibition by HONH<sub>2</sub> after an 8 minute period was 87 per cent, and by HONHR 80 per cent

† In the presence of serum albumin the inhibitions by HONH<sub>2</sub> after 8 and 30 minute periods were respectively 76 and 55 per cent, and by HONHR were 13 and 5 per cent

‡ In the presence of  $6.2 \times 10^{-8}$  M crystalline serum albumin the reversal of the inhibition by  $1 \times 10^{-5}$  M HONH<sub>2</sub> was 46 per cent, the reversal of the inhibition by  $4.58 \times 10^{-5}$  M HONHR was 19 per cent

and HONHR manifest affinities towards hemin as well. The evidence that HONH<sub>2</sub> combines with hemin was derived also from experiments in which the catalase activity of higher concentrations of hemin *per se* was inhibited 50 per cent by HONH<sub>2</sub>.

$2.72 \times 10^{-10}$  M liver catalase contains  $1.1 \times 10^{-9}$  M hemin. The inhibi-

tion of catalase was reversed 46.0 to 50.0 per cent by  $4.73 \times 10^{-6}$  M hemin. A direct comparison between the affinities of catalase hemin and the crystalline hemin towards  $\text{HONH}_2$  and  $\text{HONHR}$  is difficult to make because

TABLE III

*Reversibility of Inhibition of Liver Catalase by Hydroxylamine and p-Hydroxylaminobenzenesulfonamide (HONHR) in Presence of Hemin*

Reaction period 150 minutes.

Reaction systems	O <sub>2</sub>	H <sub>2</sub> O <sub>2</sub>	Inhibition		Reversal	
			Iodo-metrically	Mano-metrically	Iodo-metrically	Mano-metrically
2.72 × 10 <sup>-10</sup> M liver catalase (hemin content = 1.1 × 10 <sup>-9</sup> M)						
	c.mm.	micro-moles	per cent	per cent	per cent	per cent
1. No inhibitor .....	545	1.1				
2. " " + 9.5 × 10 <sup>-7</sup> M hemin....	520	3.6				
3. " " + 4.73 × 10 <sup>-6</sup> " " ....	513	3.8				
4. 1 × 10 <sup>-5</sup> M HONH <sub>2</sub> .....	92	40.5	84	83*		
5. 1 × 10 <sup>-5</sup> " " + 9.5 × 10 <sup>-7</sup> M hemin.....	164	33.4	71	70*	16	16
6. 1 × 10 <sup>-5</sup> M HONH <sub>2</sub> + 4.73 × 10 <sup>-6</sup> M hemin.....	299	20.6	45	42*	46	50
7. 4.58 × 10 <sup>-5</sup> M HONHR .....	152	34.0	70	72*		
8. 4.58 × 10 <sup>-5</sup> " " + 9.5 × 10 <sup>-7</sup> M hemin.....	166	32.3	67	68*	4	6
9. 4.58 × 10 <sup>-5</sup> M HONHR + 4.73 × 10 <sup>-6</sup> M hemin.....	384	14.8	33	26*	52	64
Catalase absent. Controls						
10. 1 × 10 <sup>-5</sup> M HONH <sub>2</sub> .....	6	48.3				
11. 1 × 10 <sup>-5</sup> " " + 9.5 × 10 <sup>-7</sup> M hemin.....	6	47.3				
12. 1 × 10 <sup>-5</sup> M HONH <sub>2</sub> + 4.73 × 10 <sup>-6</sup> M hemin.....	29	45.8				
13. 4.58 × 10 <sup>-5</sup> M HONHR.....	8	49.0				
14. 4.58 × 10 <sup>-5</sup> " " + 9.5 × 10 <sup>-7</sup> M hemin.....	7	48.3				
15. 4.58 × 10 <sup>-5</sup> M HONHR + 4.73 × 10 <sup>-6</sup> M hemin.....	33	44.8				

\* In Systems 4 to 9, the inhibitions after 8 minutes were respectively 83, 79, 64, 66, 67, and 29 per cent.

of the fact that they combine with both the protein and the hemin components of catalase. From the fact, however, that  $6.2 \times 10^{-8}$  M crystalline serum albumin and  $4.73 \times 10^{-6}$  M hemin reverse the inhibition of catalase

to the same degree, it would appear that  $\text{HONH}_2$  manifests about 100-fold greater affinity towards protein than it does towards hemin.

Stern (6) found that hydroxylamine did not bring about a change in the spectrum of catalase. He stated that the stability of the enzyme spectrum towards hydroxylamine is of interest in view of the fact that this substance inhibits the activity of the enzyme. Keilin and Hartree (7) found likewise that  $\text{HONH}_2$  does not appreciably change its color or the general pattern of its absorption spectrum.

Smythe (8) stated that hydroxylamine inhibited yeast fermentation. Sevag and Shelburne (1) found that hydroxylamine (likewise  $\text{HONHR}$ ) inhibited aerobic bacterial respiration and the anaerobic glycolysis of glucose by bacteria. Frank and Gaffron (9) stated that hydroxylamine inhibited the photosynthetic reactions by plants and bacteria. There were lacking, however, definite data as to the point of attack by  $\text{HONH}_2$  on the above enzyme systems. The results of the present study show that hydroxylamine and its derivatives are capable of combining both with the heme type of enzyme systems and those enzyme systems which do not involve heme groups.

#### SUMMARY

The inhibition of blood and crystalline liver catalase by hydroxylamine and *p*-hydroxylaminobenzenesulfonamide is completely reversible in the presence of serum, crystalline serum albumin, and hemin.

Hydroxylamine and *p*-hydroxylaminobenzenesulfonamide combine with both hemin and crystalline serum albumin free from hemin. These inhibitors are, therefore, capable of inhibiting both the heme and non-heme type of enzyme systems.

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# THE HISTIDINE CONTENT OF THE HEMOGLOBIN OF MAN AND OF THE HORSE AND SHEEP, DETERMINED WITH THE AID OF 3,4-DICHLOROBENZENESULFONIC ACID

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It has recently been shown that 3,4-dichlorobenzenesulfonic acid forms a salt with histidine that has excellent properties for the isolation of this amino acid from protein hydrolysates (1). The reagent is one of the large group of sulfonic acids being studied by Bergmann and his collaborators with the object of developing new procedures for the determination of the amino acid composition of proteins (2). Bergmann's chief interest is in methods of the solubility product type (3) for which reagents that yield salts of a moderate degree of solubility are desirable. The marked insolubility of some of these new salts of the amino acids suggests, however, that in certain cases gravimetric determination may prove feasible, and a method of this type in which 3,4-dichlorobenzenesulfonic acid is employed for the determination of histidine in hemoglobin is described in the present paper.

The tendency of the monosulfonate of leucine to separate from the hydrolysate of hemoglobin together with the histidine salt, when an excess of 3,4-dichlorobenzenesulfonic acid is added, indicated that there was little hope of finding conditions under which the basic amino acid alone could be precipitated and purified with analytical rigor; attention was accordingly turned to the use of the reagent to determine histidine in the mixture of amino acids that is precipitated from the hydrolysate by silver salts at neutral reaction. Although histidine is by far the major component, the exact composition is not known, save that cystine or cysteine is almost invariably present (4). Hitherto, purification of the histidine has usually been carried out by precipitation with mercuric sulfate (5), an operation which, although quantitative or nearly so (6) with respect to the isolation of histidine, presents technical difficulties. It has been found that the sulfonic acid reagent serves admirably for the separation of histidine from this mixture in a form suitable for weighing, and the determination of histidine in hemoglobin is thereby materially simplified. The conditions under which the technique can be applied in the case of proteins of low histidine content remain to be determined.

## EXPERIMENTAL

The preparation of the hydrolysates is carried out exactly as for the determination of arginine (7). Approximately 25 gm. of hemoglobin are taken for each set of analyses and the hydrolysate is made to 250 ml. Of this, four 1 ml. aliquots are used for the determination of nitrogen by the macro-Kjeldahl method (8 hours digestion time), and the exact quantity of protein present is calculated from the corrected nitrogen content of the dry preparation. The remainder of the hydrolysate is decolorized by treatment with norit, again brought to 250 ml., and four 50 ml. aliquots, or the equivalent of approximately 5 gm. of protein each, are taken for analysis. This yields a convenient amount of histidine disulfonate.

*Preparation of Histidine Solution*—To the 50 ml. aliquot, in a 500 ml. centrifuge bottle, 20 per cent aqueous silver nitrate solution is added with continuous agitation. The reagent is run in from a burette until a drop of the carefully stirred solution placed on a watch-glass yields a brown precipitate when a drop of cold saturated barium hydroxide solution is added; from 60 to 70 ml. are required as a rule. The test samples are washed back quantitatively into the main solution. The solution is then brought to pH 7.4 by the addition of 1.0 N sodium hydroxide. Slow addition and thorough agitation are essential in order to avoid the production of high local alkalinity and consequent precipitation of arginine silver. The end-point is judged by adding a few drops of 0.04 per cent bromothymol blue solution to a small sample after this has been centrifuged clear. The color is compared with a suitable buffer solution to which the same amount of indicator has been added. The test samples are quantitatively returned as before.

The precipitate of silver chloride and histidine silver obtained from hemoglobin hydrolysates is dark in color, owing to the presence of ferrous hydroxide. It is centrifuged and the solution is decanted through a thin layer of paper pulp on a  $\frac{1}{4}$  inch Hirsch funnel in order to retain the trace of precipitate that may be disturbed, and is washed twice successively by being thoroughly stirred with about 250 ml. of cold water and centrifuged. The washings are decanted through the same filter, and the filtrate is acidified with hydrochloric acid and set aside for the recovery of silver.<sup>1</sup>

The precipitate is again suspended by being stirred with warm water, and 3 ml. of concentrated hydrochloric acid are added. The bottle is stoppered and shaken to insure complete decomposition of the histidine compound, and is centrifuged. The clear solution is poured through the

<sup>1</sup> Owing to the presence of sodium, the filtrate cannot be used for the determination of arginine by the diflavanate method. The use of barium hydroxide for the neutralization is technically less convenient and also involves the risk of contamination of the histidine fraction with barium which forms an insoluble sulfonate.

same Hirsch funnel into a clean filter flask, care being taken that all particles of precipitate on the paper are moistened with the acid solution. If the filtrate is turbid, it is poured through the filter a second time. The silver chloride is then washed three times successively with 250 ml. of hot water containing a few drops of hydrochloric acid, being shaken thoroughly each time. The centrifuged wash solutions are likewise decanted through the funnel. The silver chloride is set aside for recovery.

The acid extract, which contains all of the histidine of the protein sample, is evaporated to complete dryness *in vacuo* in a 3 liter flask to remove excess of hydrochloric acid. The residue is dissolved in a little hot water and, in order to remove the trace of silver chloride which is usually present, is transferred quantitatively through a thick pad of paper pulp in a 2 inch Hirsch funnel directly into a 500 ml. flask.<sup>2</sup> The solution is then concentrated *in vacuo* to small volume and washed quantitatively into a beaker. The final volume should be close to 20 ml. If too much wash water has been used, the solution is evaporated on the steam bath to this volume.

*Isolation of Histidine Disulfonate*—Sufficient 3,4-dichlorobenzenesulfonic acid to provide approximately 4 moles for the assumed amount of histidine present is added in solid form (6.8 gm. of sulfonic acid dihydrate per gm. of histidine), and the solution is warmed until the precipitated histidine salt is dissolved. In order to promote the formation of large, easily washed crystals, it is best to allow the solution to cool slowly to room temperature, before placing the beaker in the refrigerator. The solution is chilled for 24 to 40 hours and the crystals are transferred, by the repeated use of the cold mother liquor, to a weighed sintered glass crucible. This is best accomplished by the use of the filtration apparatus equipped with a suitable holder for the crucible. The filtrate is received in a small beaker and the entire apparatus is preferably chilled before use. After transfer is completed, the crystals are carefully washed with a total of from 3 to 5 ml. of a cold 4 per cent solution of the sulfonic acid reagent, and are sucked dry. The crucible is wiped and is then dried for a few hours in a vacuum desiccator over sulfuric acid. To remove the adhering trace of sulfonic acid, the crystals are washed with 30 ml. of ether added in three portions and allowed to run through slowly. The crucible is finally dried at 105° to constant weight. The factor to convert the weight of disulfonate to weight of histidine is 0.2548.

For the identification of the crystals, reliance has been placed in the

<sup>2</sup> For these operations the distillation apparatus described a few years ago (8) is very convenient, as it permits the use of ring neck flasks. The filtration is carried out in a dome-covered Pyrex distillation apparatus (Central Scientific Company, catalogue No. 12910) with a Hirsch funnel and suction line mounted in a 2-hole stopper.

## EXPERIMENTAL

The preparation of the hydrolysates is carried out exactly as for the determination of arginine (7). Approximately 25 gm. of hemoglobin are taken for each set of analyses and the hydrolysate is made to 250 ml. Of this, four 1 ml. aliquots are used for the determination of nitrogen by the macro-Kjeldahl method (8 hours digestion time), and the exact quantity of protein present is calculated from the corrected nitrogen content of the dry preparation. The remainder of the hydrolysate is decolorized by treatment with norit, again brought to 250 ml., and four 50 ml. aliquots, or the equivalent of approximately 5 gm. of protein each, are taken for analysis. This yields a convenient amount of histidine disulfonate.

*Preparation of Histidine Solution*—To the 50 ml. aliquot, in a 500 ml. centrifuge bottle, 20 per cent aqueous silver nitrate solution is added with continuous agitation. The reagent is run in from a burette until a drop of the carefully stirred solution placed on a watch-glass yields a brown precipitate when a drop of cold saturated barium hydroxide solution is added; from 60 to 70 ml. are required as a rule. The test samples are washed back quantitatively into the main solution. The solution is then brought to pH 7.4 by the addition of 1.0 N sodium hydroxide. Slow addition and thorough agitation are essential in order to avoid the production of high local alkalinity and consequent precipitation of arginine silver. The end-point is judged by adding a few drops of 0.04 per cent bromothymol blue solution to a small sample after this has been centrifuged clear. The color is compared with a suitable buffer solution to which the same amount of indicator has been added. The test samples are quantitatively returned as before.

The precipitate of silver chloride and histidine silver obtained from hemoglobin hydrolysates is dark in color, owing to the presence of ferrous hydroxide. It is centrifuged and the solution is decanted through a thin layer of paper pulp on a 4 inch Hirsch funnel in order to retain the trace of precipitate that may be disturbed, and is washed twice successively by being thoroughly stirred with about 250 ml. of cold water and centrifuged. The washings are decanted through the same filter, and the filtrate is acidified with hydrochloric acid and set aside for the recovery of silver.<sup>1</sup>

The precipitate is again suspended by being stirred with warm water, and 3 ml. of concentrated hydrochloric acid are added. The bottle is stoppered and shaken to insure complete decomposition of the histidine compound, and is centrifuged. The clear solution is poured through the

<sup>1</sup> Owing to the presence of sodium, the filtrate cannot be used for the determination of arginine by the diflavanate method. The use of barium hydroxide for the neutralization is technically less convenient and also involves the risk of contamination of the histidine fraction with barium which forms an insoluble sulfonate.

and it was concluded that this figure might properly be used to correct determinations of histidine in hemoglobin carried out under essentially similar conditions. It is probable that this factor should be redetermined if variations in the conditions of the analysis are made, and especially if the method is applied to a protein of low histidine yield.

*Histidine of Horse Hemoglobin*—A preparation of horse hemoglobin made in Professor Cohn's laboratory and examined some years ago for basic amino acids (6) was employed; the earlier figure for histidine, obtained by the silver precipitation method applied to 200 gm. of the protein, was 7.64 per cent. The data of six determinations shown in Table II give an average of 7.54 per cent. Divided by the factor 0.984, this amounts

TABLE I

*Recovery of Histidine Added to Hydrolysate of Zein from Which Histidine and Arginine Had Previously Been Removed*

Each aliquot represented approximately 5 gm. of zein and contained histidine added as the monohydrochloride monohydrate in such amount as to simulate the conditions found in hydrolysates of hemoglobin. Blank determinations carried out without addition of histidine gave no disulfonate.

Histidine added	Histidine found		Decomposition point of disulfonate	Nitrogen of disulfonate, theory 6.90
gm.	gm.	per cent	°C.	per cent
0.3550	0.3495	98.47	273-274	6.94
	0.3538	99.65	272	6.87
	0.3469	97.74	273-275	6.95
0.3388	0.3312	97.77	279-280	6.95
	0.3348	98.83	278-279	6.88
	0.3327	98.18	280	6.93
Average		98.44		

to  $7.66 \pm 0.16$  per cent. The close agreement with the earlier result is probably less significant than would appear, since the precision of these determinations is not all that could be desired. The values were obtained during the course of developing the technique, and it is possible that the correct value is a little greater than this average. For calculation of the data the nitrogen content of dry horse hemoglobin was taken as 16.72 per cent, based upon the earlier value of 16.69 per cent in dry protein that contained 0.64 per cent of ash instead of the theoretical 0.47 per cent.

*Histidine of Human Hemoglobin*—Preparations of human hemoglobin, each of which had been crystallized three times and subsequently coagulated in hot water and washed with water, alcohol, and ether, were made available through the courtesy of Professor Edwin J. Cohn and of Professor

TABLE II  
Histidine Yielded by Hemoglobin

Hemoglobin	Weight of sample	Histidine found		Decomposition point of disulfonate	Nitrogen of disulfonate; theory 6.90
		gm.	gm.		
Horse	5.484	0.4119	7.51	278-279	6.86
		0.4166	7.60	278-279	6.87
	4.728	0.3610	7.65	277-278	6.86
		0.3656	7.75	278-279	6.82
		0.3481	7.38	277-278	6.86
		0.3469	7.35	277-278	7.02
Average.....		7.54 ± 0.16			
Corrected average.....		7.66 ± 0.16			
Humnn. Cohn preparation	4.802	0.3832	7.98	278-280	7.06
		0.3796	7.91	279-280	6.94
		0.3666	(7.64)*	278-279	6.88
		0.3695	(7.70)*	279-281	6.87
	5.172	(0.4215)†	8.04	278-280	6.76
		0.4130	7.99	278-280	6.78
Cannan preparation	5.548	0.4135	8.00	276	6.84
		0.4121	7.97	276-277	6.85
		0.4394	7.92	278-279	6.88
		0.4440	8.00	275	6.87
	4.586	0.4421	7.97	273	6.86
		0.4408	7.95	272-273	
		0.3628‡	7.92	276-277	6.89
		0.3656‡	7.97	277-278	6.90
	0.3656‡	7.97	279-280		
	0.3623‡	7.90	278	6.95	

\* Omitted from the mean; see the text.

† This quantity leads to a histidine value of 8.15 per cent. The specimen was found to yield 0.244 per cent of ferric oxide. Assuming that the contaminant was ferrous disulfonate, it therefore contained 1.22 per cent of impurity. The percentage yield of histidine has accordingly been corrected.

‡ Although apparently pure, these four samples were recrystallized twice successively. The average loss of weight for the first recrystallization was 0.0417 gm. of disulfonate, for the second 0.0196 gm. The percentage yields given refer to the histidine disulfonate found after the first recrystallization corrected by 0.0196 gm., it being assumed that the difference (0.0221 gm.) represents impurities (e.g. ferrous salt) removed by this step. The average yield of histidine calculated from the corrected results after one recrystallization was 7.94 per cent; that calculated after two recrystallizations and corrected a second time was likewise 7.94 per cent. The decomposition points and nitrogen determinations refer to the preparations after two recrystallizations.

TABLE II.—*Concluded*

Hemoglobin	Weight of sample	Histidine found		Decomposition point of disulfonate	Nitrogen of disulfonate, theory 6.90
		gm	per cent	°C	per cent
Average (6 determinations, Cohn preparation)			7.98 ± 0.04		
Corrected average			8.11 ± 0.04		
Average (8 determinations, Cannan preparation)			7.95 ± 0.04		
Corrected average			8.08 ± 0.04		
Average (14 determinations, both preparations)			7.96 ± 0.04		
Corrected average			8.09 ± 0.04		
Sheep	5.592	0.4070	7.30	277-279	6.76
		0.4036	7.24	277-278	6.80
Average			7.27		
Corrected average			7.38		

R. K. Cannan. The methods employed for crystallization were entirely different in the two cases. Cohn's preparation contained 15.05 per cent of nitrogen, 11.35 per cent of moisture, and 0.33 per cent of ash (0.37 per cent corrected for moisture). The nitrogen content of the dry material was therefore 17.00 per cent if no allowance is made for the slight deficiency of ash from the theoretical 0.47 per cent. Cannan's sample contained 15.62 per cent of nitrogen, 8.15 per cent of moisture, and 0.41 per cent of ash (0.45 per cent corrected for moisture), or 16.90 per cent of nitrogen on the same basis. It is possible that the slightly low proportion of ash in each case was due to loss of a little heme during the coagulation and washing operations. For purposes of calculation, the nitrogen content of dry human hemoglobin was taken as the average of the two preparations, 16.95 per cent, without further correction for the ash content.

Data from four separate sets of four determinations each are shown in Table II. A statistical analysis of the sixteen values taken in pairs was made by Dr. C. I. Bliss of this Station, which showed that the second pair of analyses of Cohn's sample should be eliminated in computing the mean. The average of the fourteen acceptable values is 7.96 per cent, which, divided by the factor 0.984, gives  $8.09 \pm 0.04$  per cent as the most probable value for the histidine yielded by these preparations of human hemoglobin. It was found necessary to correct one determination for contamination with iron (see foot-note to Table II), since analysis showed

the presence of a significant proportion of ferric oxide ash in the specimen. The last four values illustrate the effect of recrystallization of the disulfonate and of correction for loss in this operation.

*Histidine of Sheep Hemoglobin*—A preparation made by Professor A. White in 1933 (9) was found to contain 15.60 per cent of nitrogen, 6.58 per cent of moisture, and 0.72 per cent of ash. The nitrogen content of the dry protein calculated with a theoretical ash content of 0.47 per cent was therefore 16.85 per cent. A previous analysis by Professor White gave 16.83 per cent.

Owing to the limited quantity of protein available, only one pair of histidine determinations was carried out, the balance of the hydrolysate being used for the determination of arginine. The results shown in Table II lead to a corrected value of 7.38 per cent for the histidine yielded by this hemoglobin.

TABLE III  
*Histidine Yielded by Edestin*

Weight of sample	Histidine found		Decomposition point of disulfonate	Nitrogen of disulfonate
gm.	gm.	per cent	°C.	per cent
4.416	0.1149	2.60	278-279	6.93
	0.1154	2.62	277-278	6.93
4.719	0.1223	2.59	279-280	6.99
	0.1247	2.64	278-280	6.94
	0.1249	2.65	280	6.91
	0.1285	2.72	277-278	6.70
Average		2.64 ± 0.05		

*Histidine of Edestin*—Preliminary experiments were carried out with edestin, these being chiefly in connection with controls on the technique. The results are given here only to illustrate the reproducibility but with reservation as to the accuracy. The data from six analyses are shown in Table III but, although the decomposition points and nitrogen values are satisfactory, all of the preparations were dark in color and a moderate proportion of impurity was doubtless present. No arginine could be detected by means of flavianic acid in the mother liquors from which these preparations of histidine salt had been crystallized, and the separation of histidine from arginine had accordingly been successful in these particular cases. In a few other tests, however, arginine was detected in small amounts, presumably because the reaction had inadvertently been carried a little too far to the alkaline side during the precipitation of the silver compound. Contamination with arginine renders it difficult to obtain a pure preparation of the histidine salt under analytical conditions.



The factor by which the result of a determination of histidine in a protein that yields only a small proportion of this amino acid should be corrected was not determined and no correction has been applied to these data. The histidine salt was crystallized from a smaller volume than that employed in the hemoglobin analyses, but suitable general conditions for analyses of this type remain to be established.

The present result is considerably higher than the value of 2.08 per cent obtained some years ago in this laboratory by the large scale silver precipitation method (10). This early value has been confirmed by small scale silver precipitation methods both by Tristram (11) and by Block (12), but there is good reason to believe that it is appreciably too low. Chibnall<sup>3</sup> has recently obtained the value 2.41 per cent in an extremely careful analysis and there seems some likelihood that further study of the application of the present method to proteins of low histidine content may reconcile this discrepancy.

*Arginine of Hemoglobin*—The determination by the diflavianate procedure (7) of arginine yielded by hemoglobin is especially difficult, because, if sufficient flavianic acid is added to provide for an adequate excess of reagent, an appreciable quantity of histidine diflavianate is precipitated with the arginine salt. Accordingly two and possibly more recrystallizations of the arginine monoflavianate may be required in order to eliminate contamination. Analysis of Cohn's preparation of human hemoglobin gave three values of 4.22, 4.22, and 4.20 per cent of arginine; a composite sample of the monoflavianate from these determinations contained 6.54 per cent of sulfur (theory 6.56 per cent). The average of 4.21 per cent is therefore the most probable value for the arginine yielded by this protein.

Duplicate determinations on sheep hemoglobin gave 3.92 and 3.87 per cent of arginine after three recrystallizations of the arginine monoflavianate. The sulfur content of a composite sample of the monoflavianate was 6.62 per cent.

#### DISCUSSION

The results of the present analyses, together with a recent determination of arginine yielded by the same sample of horse hemoglobin (7), are collected in Table IV. It is clear that there are differences in the basic amino acid composition of these hemoglobins and the present evidence is thus in accordance with earlier data for sulfur content as well as, for example, with the crystallographic data of Reichert and Brown (13) and the spectrographic data of Winegarden and Borsook (14).

That the hemoglobins of different species differ in chemical composition from each other has long been known. Zinoffsky (15) determined the

<sup>3</sup> Personal communication; Chibnall's data are shortly to be published.

iron and sulfur content of horse hemoglobin in 1886 and Jaquet (16), a little later, that of the dog and the hen with great accuracy. Although the iron content appeared to be uniform, there were considerable differences in the sulfur. Gamgee (17) in 1898 reviewed all available data on these and other hemoglobins and pointed out that "there is such a difference in the ratio of S:Fe in the haemoglobin of certain animals as renders it . . . certain that . . . the albuminous moiety of the complex molecule differs." In recent years, the iron and sulfur content of a number of hemoglobins, including that of man, has been reexamined by a group of Hungarian investigators (18) with complete confirmation of the early workers.

The extraordinary uniformity in the iron content of the hemoglobins of different species, namely 0.335 per cent with variation of only a few units in the third decimal place, establishes the minimal molecular weight of these proteins as a little less than 16,700. Studies of the osmotic pressure (19) and of the sedimentation constant (20) have shown that the

TABLE IV  
*Arginine and Histidine Yielded by Hemoglobins*

Hemoglobin	Arginine			Histidine		
	Horse	Human	Sheep	Horse	Human	Sheep
% of protein	3.59	4.21	3.89	7.66	8.09	7.38
N as % of protein N	6.91	7.99	7.43	12.4	12.9	11.9
Moles $\times 10^{-3}$ per gm.	20.6	24.2	22.4	49.4	52.1	47.6
“ per 66,700 gm. Calculated	13.8	16.1	14.9	32.9	34.8	31.7
Nearest integer	14	16	15	33	35	32

true molecular weight of the anhydrous hemoglobins of several species must be 4 times this quantity; *i.e.*, close to 66,700. The molecular weight of few if any proteins is known with so high a degree of accuracy.

Gamgee's inference with respect to the sulfur of hemoglobins has been confirmed by the demonstration in this laboratory (9) of differences in cystine content in the hemoglobins of the horse, sheep, and dog, and more recently by Beach and coworkers (21) and by Kuhn, Birkofer, and Quackenbush, who have extended the observations to methionine as well, in the case of a number of globins. On the other hand, close similarities with respect to the proportions of the basic amino acids and of tyrosine, tryptophane, and phenylalanine yielded by the hemoglobins of the horse, sheep, ox, and dog have been reported by Block (23), although the analytical results for the basic amino acids were admittedly "only approximate" (24).

The data in Table IV not only indicate that the arginine and histidine contents of the three hemoglobins studied differ from each other but suggest that the plan or pattern of the distribution of the basic amino acids in the

molecules of these proteins may also be different. Too little is known at the present time regarding the details of protein constitution to discuss this conclusion with profit, but it would seem that if the distribution of the basic amino acids, that is of the positively charged groups of the protein molecule, has anything to do with the function of hemoglobin in the animal, the structural problem involved in providing for this function has been solved in somewhat different ways in different species.

#### SUMMARY

Advantage is taken of the insolubility of the salt that histidine forms with 2 molecules of 3,4-dichlorobenzenesulfonic acid to determine the histidine yielded by the hemoglobins of man, and of the horse and sheep, and a suitable technique for the analysis is described. Human hemoglobin was found to yield  $8.09 \pm 0.04$  per cent, that of the horse  $7.66 \pm 0.16$  per cent, and that of the sheep close to 7.38 per cent of histidine. If it be assumed, from their uniform iron content, that these proteins possess approximately the same molecular weight, namely 66,700, the figures correspond closely to the presence, respectively, of 35, 33, and 32 histidine residues per molecule of hemoglobin.

Determinations of arginine indicated that human hemoglobin yields 4.21 per cent and that of the sheep 3.89 per cent. On the same assumption regarding molecular weight, human, horse, and sheep hemoglobins accordingly contain close to 16, 14, and 15 arginine residues respectively per molecule. The results are interpreted to indicate that these three hemoglobins differ with respect to the proportions, and possibly also with respect to the arrangement of these basic groups in the molecule.

Preliminary data on the histidine yielded by edestin suggest that this globulin yields approximately 2.6 per cent of histidine. This figure is subject to later correction.

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# SOME FACTORS THAT AFFECT THE MICROBIOLOGICAL METHOD FOR RIBOFLAVIN

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Considerable difficulty has been experienced in obtaining the correct riboflavin values for wheat flours by the Snell and Strong (1) microbiological method in this and in other laboratories (2). The values obtained are too high, especially when the flour extract is assayed at a low level. According to Bauernfeind, Sotier, and Boruff (3) fatty acids stimulate the action of *Lactobacillus casei* and the method can be improved by extracting the sample with ether. Andrews, Boyd, and Terry (4) and Scott, Randall, and Hessel (5) have observed that treatment of whole wheat flour extracts with taka-diastase removes, to a large extent, the factors which affect the assay. The possibility presented itself that the difficulty might be due to a deficiency of the basal medium and that by adding riboflavin-free extracts prepared from cereal grains these discrepancies could be avoided. Experiments accordingly were performed to investigate this possibility.

## Preparation of Material

Extracts of whole wheat flour, of rice, and of wheat bran were prepared by autoclaving these materials in 0.1 N HCl for 15 minutes at 15 pounds pressure. 15 ml. of acid were used per gm. of sample. The samples were centrifuged and the residue washed twice with small amounts of water.

In order to destroy the riboflavin, whole wheat flour extracts were adjusted to pH 11.0 with 1 N NaOH and photolyzed by exposure at approximately 33° to a 100 watt Mazda light bulb at a distance of 1 foot for 6 or 24 hours. The effect of alkalinity alone was also determined by adjusting the pH of the extract to 11.0 and placing the extract in the dark for the same period of time.

Some of the alkaline-photolyzed preparations were extracted with ether. For this operation the pH was adjusted to 3.0 and the solution (approximately 75 ml.) was shaken four times in a wide mouth bottle with 20 ml. of alcohol-free ethyl ether and about 5 ml. of petroleum ether. The petroleum ether was added to facilitate the separation of the two layers. The solution was centrifuged for a few minutes. The ether layer was drawn off with a pipette, evaporated to dryness *in vacuo*, and the residue suspended in water. Untreated acid extracts of whole wheat flour were

extracted with ether in a like manner. These extractions were carried out on the centrifuged extracts without pH adjustment (pH 1.2 to 1.4).

Concentrated extracts of rice and of wheat bran were prepared by adjusting the pH of the original acid extract to 6.7, passing the extract through a column of florasil to remove riboflavin, and concentrating the filtrate *in vacuo*.

The effect of taka-diastrase and papain was also determined. An acid extract of 5 gm. of whole wheat flour in 75 ml. of solution was adjusted to pH 5.0 and 0.1 gm. of taka-diastrase and 0.1 gm. of papain were added. Then the solution was allowed to stand overnight under toluene at a temperature of 37°.

The extracts were adjusted to pH 6.6 to 6.8 and added to assay tubes prepared according to the usual method (2), containing definite amounts of riboflavin. Then the tubes were incubated and titrated. Titration values are reported in ml. of 0.1 N acid produced by *Lactobacillus casei* in 72 hours incubation, at 37°.

### Results

The results of the addition of various extracts are presented in Table I. Alkaline-photolyzed whole wheat flour extracts, devoid of riboflavin, caused a large increase in acid production when added to tubes containing riboflavin. Thus assay tubes containing photolyzed extract equivalent to 50 mg. of whole wheat flour and 0.05  $\gamma$  of riboflavin produced 6.6 ml. of 0.1 N acid, while tubes with the equivalent amount of flour, as an untreated extract containing 0.05 to 0.07  $\gamma$  of riboflavin, produced only 2.7 ml. The response was more exaggerated with 0.05  $\gamma$  than with 0.2  $\gamma$  of riboflavin, and was greater with 24 hours exposure to light and alkali than with 6 hours. Material removed by ether extraction stimulated acid production but the enhancing value of the photolyzed solution was reduced only slightly by the extraction with ether. An alkalinized extract which contained some riboflavin exerted a smaller stimulating effect than an alkaline-photolyzed solution. It appears then that photolysis at pH 11.0 produces substances that stimulate the acid production of *Lactobacillus casei*.

Wheat flour extracts which had been passed through florasil at pH 6.7 to remove the riboflavin did not contain the substance activated by photolysis but did contain a factor which exerts some stimulation. This was shown when extracts equivalent to 100 mg. of flour, passed through the florasil, gave a response of 3.0 ml. of acid when added to 0.05  $\gamma$  of riboflavin, whether photolyzed or not.

Concentrated extracts of rice bran, wheat bran, or whole wheat flour passed through florasil to remove the riboflavin and then added to assay tubes containing 1.0  $\gamma$  of riboflavin were found to produce up to 20.6 ml.

of 0.1 N acid, which is well beyond the amount of 9 to 10 ml., previously considered the maximum. The effect of adding various amounts of a riboflavin-free concentrate of rice bran to different levels of riboflavin was observed. Increasing the amount of concentrate from 20 to 200 mg. equivalents of rice bran at the 0.05  $\gamma$  level increased the titration from 2.5

TABLE I  
*Stimulation of Acid Production by Various Additions*

	Substance equiva- lent per tube	Acid (0.1 N) produced			
		0.00 $\gamma$	0.05 $\gamma$	0.20 $\gamma$	1.0 $\gamma$ *
	mg	ml	ml	ml	ml
Standard riboflavin	0	0 0	1 8	7 2	9 8
Whole wheat flour extract					
No treatment	50	2 7			
"    "	500				16 4
Ether extract	50	0 0	2 6	7 0	
"    "	200	0 0	3 6	8 1	
Alkaline-photolyzed 24 hrs	50	0 0	6 6	8 0	12 7
"    24    "	200	0 0	6 2	8 0	13 3
Ether extracted photolyzed solution	50	0 0	6 2	7 2	
"    "    "	200	0 0	4 8	7 9	
Ether extract of    "    "	50	0 0	2 2	6 5	
"    "    "    "    "	200	0 0	2 7	7 4	
Alkaline-photolyzed 6 hrs	50	0 0	4 1		
Alkalized 24 hrs.	50		5 6		
"    24    "	200	5 3	5 8		
Florisl filtrate	100	0 0	3 0		
"    "    photolyzed 24 hrs.	100	0 0	3 1		
Rice bran extract	20	0 0	2 5	8 6	
"    "    "	200	0 2	6 2	9 4	
"    "    "	500	0 4	6 9		15 9
"    "    "    + 50 mg photolyzed flour					
extract	500	0 4	6 6		
Rice bran extract + 125 mg photolyzed flour					
extract	500	0 4	6 0		
Wheat bran extract	500				20 6
Pyridine	25		4 5		
"	100		4 6		

\* 100 mg of dextrose were added to each tube

to 6.2 ml. of 0.1 N NaOH and at the 0.2  $\gamma$  level from 8.6 to 9.4 ml. of 0.1 N NaOH. The addition of 500 mg. equivalents at the 0.05  $\gamma$  level produced a titration of 6.9 ml.

Addition of pyridine increased the production of acid, suggesting that substances containing this ring structure stimulate acid production or possibly were deficient in the medium used.

In Table II are shown the effects of treating the extracts with taka-diastase and papain on the parts per million of riboflavin estimated from the titrations. The calculated riboflavin ranged from 1.30 to 1.68 parts per million on the untreated samples, while with the enzyme it had a wider range of from 1.08 to 1.76. The treatment with the enzyme did not improve the results.

TABLE II

*Effect of Taka-diastase and Papain Treatment on Riboflavin Assay of Whole Wheat Flour*

Substance equivalent per tube	Riboflavin	
	No treatment	Enzyme treatment
mg.	p.p.m.	p.p.m.
50	1.68	1.76
100	1.70	1.50
150	1.30	1.08
Mean . . . . .	1.56	1.45

## DISCUSSION

The above experiments show quite definitely that the addition to the basal medium of riboflavin-free extracts prepared from whole wheat flour, wheat bran, and rice bran does not eliminate the difficulties encountered in riboflavin assays of these materials. The addition of alkaline-photolyzed whole wheat flour extracts stimulated the bacteria more at low levels of riboflavin than at high levels. If the extracts were added to the basal medium, the resulting standard curve would have a decreased slope in its usable range and a small change in titration value would produce a large change in riboflavin value. The addition of rice or wheat bran concentrates to the basal medium is not feasible, since the extent of stimulation at a given level of riboflavin did not become constant until the equivalent of approximately 500 mg. of sample was added per tube (Table I). At this point the type of standard curve was no longer linear.

The ability of rice and wheat bran concentrates to stimulate acid production is possibly due to the same substance reported by Clarke *et al.* (6) which caused increased acid production in the pantothenic acid assay. However, their results failed to show its presence in wheat flour. The difference might be due to the fact that acid extracts were used in our laboratories instead of water extracts.

While alkaline-photolyzed whole wheat flour extracts produce effects similar to those of rice and wheat bran, we do not believe the same factor or factors are involved. This conclusion is based on the fact that bran



extracts retain their stimulating effect on being passed through a column of florisil, while wheat flour extracts receiving this treatment are not activated by alkaline photolysis. However, the effects of the two different extracts are not additive, suggesting that the same mechanism might be involved.

#### SUMMARY

1. Alkaline photolysis of whole wheat flour extracts produces a factor, or factors, which stimulates acid production by *Lactobacillus casei* in the microbiological method for riboflavin. The addition of such extract to the basal medium does not remedy the difficulties experienced in obtaining the true riboflavin values of cereal products.

2. Rice bran, wheat bran, and whole wheat flour contain a substance other than riboflavin and different from that mentioned above which is capable of stimulating acid production by *Lactobacillus casei* well above the present maximum. Addition of these extracts to the basal medium is not feasible, owing to inability to standardize the response when the extracts are present in the medium and to obtain a usable curve.

3. Treatment of whole wheat flour extracts with taka-diastase and papain does not destroy the stimulating factors.

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# THERMODYNAMIC PROPERTIES OF SOLUTIONS OF AMINO ACIDS AND RELATED SUBSTANCES

## VII. THE IONIZATION OF SOME HYDROXYAMINO ACIDS AND PROLINE IN AQUEOUS SOLUTION FROM ONE TO FIFTY DEGREES\*

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The present paper is a continuation of earlier studies on the effect of temperature on the thermodynamic ionization constants of amino acids (6, 5, 8). It includes measurements of solutions of *dl*-serine, *dl*-threonine, *dl*-allothreonine, *L*-hydroxyproline, and *L*-proline.

### *Methods and Materials*

The amino acids were commercial synthetic or natural products. The solubility determinations were made by equilibrating successive small portions of solvent with a relatively large quantity of amino acid in a constant temperature bath and determining the quantity of solute in the filtrate by drying to constant weight. Purity is indicated by the constant solubility of successive fractions. The amino acids were dried to constant weight over phosphorus pentoxide.

The serine was from the same lot used in an earlier investigation (7). The threonine, allothreonine, hydroxyproline, and proline were purchased from the Department of Chemistry, University of Illinois. They were recrystallized from water, alcohol, or mixtures of the two.

*dl*-Serine—Solubility, 51.6 and 51.4 gm. per 1000 gm. of water.

*dl*-Threonine—Solubility, 201.1 and 201.6 gm. per 1000 gm. of water. From an approximate determination of the density of the saturated solution the solubility was calculated to be 17.6 gm. per 100 cc. of solution, a value appreciably lower than the 20.1 gm. per 100 cc. of solution given by West and Carter (10) and the value of 19.0 gm. per 100 cc. of solution calculated from the data of Edsall (1).

*dl*-Allothreonine—Solubility, 127.9, 126.5, and 126.9 gm. per 1000 gm. of water. The solubility was calculated to be 11.7 gm. per 100 cc. of solution, appreciably lower than the value of 13.9 gm. per 100 cc. of solution found by West and Carter (10). The reason for the lower values for the

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solubilities of threonine and allothreonine is not apparent but may be associated with the difficulty encountered in getting fractions of constant solubility, several recrystallizations being necessary.

TABLE I

*Experimental Data Used in Determining First Acid Ionization Constant of Amino Acids*

Molality		Observed $E$ , corrected to 1 atmosphere $H_2$ at				
Amino acid	HCl	1.0°	12.5°	25.0°	37.5°	50.0°
<i>dl</i> -Serine						
0.03114	0.01756	0.4681	0.4696	0.4710	0.4714	0.4721
0.03977	0.02138	0.4638	0.4651	0.4658	0.4660	0.4657
0.08418	0.04610	0.4432	0.4431	0.4426	0.4417	0.4411
0.08728	0.04888	0.4407	0.4410	0.4400	0.4394	0.4380
0.1176	0.06479	0.4347	0.4340	0.4328	0.4318	0.4303
0.03972	0.02238	0.4614	0.4620	0.4629	0.4634	0.4632
0.05046	0.02645	0.4587	0.4593	0.4599	0.4601	0.4598
<i>dl</i> -Threonine						
0.03226	0.01751	0.4657	0.4671			
0.03383	0.01785	0.4659	0.4669			
0.03178	0.01789		0.4652	0.4669	0.4680	0.4683
0.03246	0.01831			0.4658	0.4667	0.4672
0.03556	0.01997			0.4632	0.4640	0.4642
0.04693	0.02702			0.4530	0.4530	0.4528
0.05893	0.03300	0.4470	0.4770	0.4469		
0.08890	0.04755	0.4386	0.4380			
<i>dl</i> -Allothreonine						
0.03221	0.01736	0.4651	0.4675	0.4695	0.4709	0.4718
0.03196	0.01864	0.4611	0.4633	0.4654	0.4663	0.4671
0.03811	0.02235	0.4557	0.4576	0.4595		
0.04195	0.02144	0.4602	0.4624	0.4642	0.4655	0.4661
0.03965	0.02240	0.4566	0.4583	0.4598	0.4607	0.4612
0.04146	0.02239			0.4612	0.4622	0.4626
0.04648	0.02629	0.4519	0.4536	0.4545	0.4552	0.4555
<i>L</i> -Hydroxyproline						
0.03167	0.01788	0.4547	0.4565	0.4581	0.4588	0.4590
0.03064	0.01777	0.4543	0.4562	0.4574		
0.04116	0.02178			0.4524	0.4530	0.4530
0.04039	0.02210	0.4487	0.4500	0.4512	0.4519	0.4521
0.05572	0.03113		0.4382	0.4389	0.4392	0.4386
0.07040	0.04120	0.4278	0.4283	0.4282	0.4278	0.4266
0.08642	0.04731	0.4256	0.4253	0.4259	0.4255	0.4241

TABLE I—Concluded

Molality		Observed $E$ , corrected to 1 atmosphere $H_2$ at				
Amino acid	HCl	1.0°	12.5°	25.0°	37.5°	50.0°
<i>l</i> -Proline						
0.02878	0.01909			0.4564	0.4576	0.4584
0.03943	0.02148	0.4528	0.4515	0.4566		
0.03887	0.02361		0.4490	0.4508	0.4518	0.4525
0.04808	0.02551			0.4515	0.4525	0.4532
0.04574	0.02751			0.4458	0.4467	0.4471
0.07064	0.04060			0.4347	0.4352	0.4350
0.08455	0.04787	0.4289	0.4294	0.4300	0.4302	0.4303
0.11340	0.06130	0.4236	0.4237	0.4241	0.4241	0.4240

*l*-Hydroxyproline—Solubility, 379.0, 378.1, and 379.5 gm. per 1000 gm. of water. This is an appreciably higher value than that of 361.1 gm. per 1000 gm. of water found by Tomiyama and Schmidt (9).

*l*-Proline—Solubility in approximately 95 per cent ethyl alcohol, 146.1, 144.9, and 145.1 gm. per 1000 gm. of solvent.

The hydrochloric acid was prepared from the constant boiling mixture. The sodium hydroxide was prepared by adding a centrifuged 50 per cent solution to freshly boiled distilled water. Almost identical values were obtained by standardization against potassium acid phthalate and against the hydrochloric acid solution. The sodium chloride solution was prepared from a purified sample that had been dried by heating in a platinum crucible. The electrodes were prepared and the cells were filled as described in an earlier report (8).

#### RESULTS AND DISCUSSION

For the first acid constant buffer solutions of amino acid and hydrochloric acid were measured in cells without liquid junction whose electromotive force is related to the first acid ionization constant of the amino acid by the equation

$$pK_1 - \log \frac{\gamma_{HAH^+}}{\gamma_H \cdot \gamma_{HA^+}} = \frac{(E - E_0)nF}{2.303RT} + \log m_{Cl^-} + \log \gamma_H \cdot \gamma_{Cl^-} + \log \frac{m_{Cl^-} - m_{H^+}}{m_A - m_{Cl^-} + m_{H^+}} \quad (1)$$

where A represents the amino acid in all forms. Values for  $pK_1$  are the intercepts at 0 ionic strength obtained from a plot of the right-hand side of Equation 1 against the ionic strength. For the second acid constant buffer solutions of amino acid, sodium hydroxide, and sodium chloride

TABLE II

*Experimental Data Used in Determining Second Acid Ionization Constant of Amino Acids*

Molality			Observed $E$ , corrected to 1 atmosphere $H_2$ at				
Amino acid	NaOH	NaCl	1.0°	12.5°	25.0°	37.5°	50.0°
<i>dl-Serine</i>							
0.01622	0.00795	0.00706	0.8893	0.8914	0.8930	0.8938	0.8940
0.02018	0.01141	0.01001	0.8882	0.8903	0.8917	0.8926	0.8930
0.02287	0.01293	0.01225	0.8837	0.8856	0.8869	0.8875	0.8873
0.03223	0.01534	0.01482	0.8707	0.8721	0.8729	0.8732	0.8732
0.06125	0.03090	0.02998	0.8568	0.8573	0.8574	0.8565	0.8551
0.05952	0.03119	0.03108	0.8583	0.8586	0.8588	0.8583	0.8571
0.06026	0.03187	0.03148	0.8582	0.8588	0.8587	0.8579	0.8568
<i>dl-Threonine</i>							
0.01551	0.00798	0.00791	0.8826	0.8848	0.8867	0.8881	0.8889
0.01506	0.00753	0.00918	0.8773	0.8795	0.8812	0.8824	0.8832
0.02051	0.00972	0.00936	0.8748	0.8768	0.8982	0.8792	0.8801
0.01954	0.01043	0.01036	0.8779	0.8800	0.8816	0.8827	0.8833
0.02325	0.01274	0.01284	0.8747	0.8766	0.8782	0.8790	0.8796
0.04837	0.02317	0.02182	0.8563		0.8579	0.8585	0.8573
0.04365	0.02295	0.02211	0.8601	0.8613	0.8621	0.8623	0.8623
0.02132	0.01155	0.03374	0.8516	0.8526	0.8530	0.8527	0.8519
0.06177	0.03120	0.03084	0.8509	0.8516	0.8520	0.8513	0.8509
<i>dl-Allothreonine</i>							
0.01558	0.00823	0.00683	0.8881	0.8899	0.8915	0.8923	0.8929
0.01433	0.00822	0.00802	0.8884	0.8903	0.8917	0.8926	0.8929
0.01849	0.01049	0.01046	0.8820	0.8836	0.8848	0.8852	0.8850
0.02519	0.01307	0.01207	0.8736	0.8750	0.8758	0.8760	0.8754
0.01971	0.01050	0.03497	0.8498	0.8501	0.8497	0.8489	0.8477
<i>l-Hydroxyproline</i>							
0.01649	0.00870	0.00776	0.9118	0.9167	0.9218	0.9259	0.9287
0.01565	0.00880	0.00810		0.9188	0.9238	0.9279	0.9314
0.01880	0.01055	0.01019	0.9090	0.9138	0.9185	0.9225	0.9256
0.02590	0.01426	0.01165	0.9049	0.9095	0.9143	0.9181	0.9212
0.03010	0.01638	0.01514	0.8985	0.9029	0.9072	0.9108	0.9136
0.03918	0.02326	0.02181	0.8948	0.8991	0.9032	0.9064	0.9091
<i>l-Proline</i>							
0.02604	0.01277	0.01221	0.9530	0.9582	0.9628	0.9664	0.9692
0.02462	0.01289	0.01184	0.9564	0.9616	0.9664	0.9700	0.9726
0.02963	0.01635	0.01547	0.9530	0.9581	0.9628	0.9663	0.9689
0.04351	0.02322	0.02191	0.9440	0.9487	0.9531	0.9561	0.9585
0.04288	0.02354	0.02171	0.9453	0.9500	0.9544	0.9574	0.9595

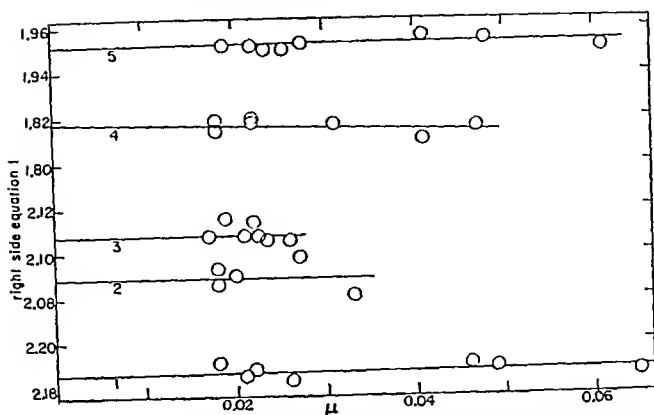


FIG. 1. A plot of the right-hand side of Equation 1 against the ionic strength. The intercept at 0 ionic strength is  $pK_1$ , at  $25^\circ$ . Curve 1 represents *dl*-serine; Curve 2, *dl*-threonine; Curve 3, *dl*-allothreonine; Curve 4, *l*-hydroxyproline; Curve 5, *l*-proline.

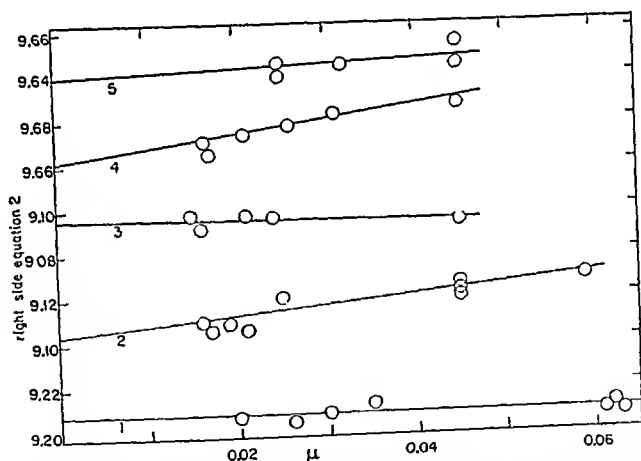


FIG. 2. A plot of the right-hand side of Equation 2 against the ionic strength. The intercept at 0 ionic strength is  $pK_2$ , at  $25^\circ$ . Curve 1 represents *dl*-serine; Curve 2, *dl*-threonine; Curve 3, *dl*-allothreonine; Curve 4, *l*-hydroxyproline; Curve 5, *l*-proline.

were measured in a cell whose electromotive force is related to the second ionization constant of the amino acid by the equation

$$pK_2 + \log \frac{\gamma_{A^-}}{\gamma_{HA} \gamma_{Cl^-}} = \frac{(E - E_0)nF}{2.303RT} + \log m_{Cl^-} + \log \frac{m_A - m_{NaOH} + m_{OH^-}}{m_{NaOH} - m_{OH^-}} \quad (2)$$

Values for  $pK_2$  are the intercepts at 0 ionic strength obtained from a plot of the right-hand side of Equation 2 against the ionic strength. Details

TABLE III

*Thermodynamic Functions for Ionization of Hydroxyamino Acids and Proline*

	1.0°	12.5°	25.0°	37.5°	50.0°
<i>dl</i> -Serine					
$pK_1$	2.296	2.232	2.186	2.154	2.132
" *	2.294	2.234	2.184	2.150	2.132
$\Delta F_1$	2887	2919	2980	3058	3153
$\Delta H_1$	1981	1721	1366	932	411
$\Delta S_1$	-3.3	-4.2	-5.4	-6.8	-8.5
<i>dl</i> -Threonine					
$pK_1$	2.200	2.132	2.088	2.070	2.055
" *	2.194	2.140	2.096	2.068	2.062
$\Delta F_1$	2752	2796	2859	2939	3048
$\Delta H_1$	1823	1549	1180	728	191
$\Delta S_1$	-3.4	-4.4	-5.6	-7.1	-8.8
<i>dl</i> -Allothreonine					
$pK_1$	2.178	2.138	2.108	2.090	2.086
" *	2.180	2.137	2.105	2.089	2.088
$\Delta F_1$	2733	2792	2871	2970	3088
$\Delta H_1$	1478	1176	772	287	-287
$\Delta S_1$	-4.6	-5.7	-7.0	-8.6	-10.4
<i>l</i> -Hydroxyproline					
$pK_1$	1.900	1.850	1.818	1.798	1.786
" *	1.897	1.851	1.815	1.796	1.789
$\Delta F_1$	2378	2420	2476	2552	2624
$\Delta H_1$	1602	1310	918	446	-115
$\Delta S_1$	-2.8	-3.9	-5.2	-6.8	-8.5
<i>l</i> -Proline					
$pK_1$	2.011	1.964	1.952	1.950	1.958
" *	2.004	1.970	1.952	1.949	1.960
$\Delta F_1$	2514	2574	2663	2770	2898
$\Delta H_1$	1149	780	342	-181	-746
$\Delta S_1$	-5.0	-6.3	-7.8	-9.5	-11.3

\*  $pK$  calculated by the equation of Harned and Embree (3).

of the calculations and the assumptions necessary are given in an earlier paper (8).



Tables I and II include the compositions of the solutions and the observed potentials. Figs. 1 and 2 illustrate the results obtained at 25°. A

TABLE IV  
Thermodynamic Functions for Ionization of Hydroxyamino Acids and Proline

	1.0°	12.5°	25.0°	37.5°	50°
<i>dl</i> -Serine					
$pK_2$	9.880	9.542	9.208	8.904	8.628
" *	9.878	9.543	9.205	8.903	8.630
$\Delta F_2$	12380	12480	12550	12660	12750
$\Delta H_2$	10450	10490	10405	10200	9840
$\Delta S_2$	-7.0	-6.9	-7.2	-7.9	-9.1
<i>dl</i> -Threonine					
$pK_2$	9.748	9.420	9.100	8.812	8.548
" *	9.747	9.422	9.100	8.810	8.550
$\Delta F_2$	12260	12315	12410	12520	12640
$\Delta H_2$	10075	10085	9960	9712	9320
$\Delta S_2$	-8.0	-7.8	-8.2	-9.0	-10.3
<i>dl</i> -Allothreonine					
$pK_2$	9.774	9.432	9.096	8.796	8.520
" *	9.769	9.439	9.096	8.795	8.522
$\Delta F_2$	12250	12535	12400	12490	12600
$\Delta H_2$	10430	10465	10380	10150	9800
$\Delta S_2$	-6.6	-7.2	-6.8	-7.5	-8.7
<i>l</i> -Hydroxyproline					
$pK_2$	10.274	9.958	9.662	9.394	9.138
" *	10.273	9.964	9.660	9.388	9.146
$\Delta F_2$	12880	13020	13160	13350	13525
$\Delta H_2$	9585	9543	9385	9080	8635
$\Delta S_2$	-12.0	-12.2	-12.7	-13.7	-15.1
<i>l</i> -Proline					
$pK_2$	11.296	10.972	10.640	10.342	10.064
" *	11.305	10.973	10.640	10.338	10.069
$\Delta F_2$	14175	14340	14510	14660	14890
$\Delta H_2$	10360	10400	10310	10085	9715
$\Delta S_2$	-13.9	-13.8	-14.1	-14.7	-16.0

\*  $pK$  calculated by the equation of Harned and Embree (3).

summary of the values for  $pK_1$  and  $pK_2$  appears in Tables III and IV. Because of the limited quantities of materials and the difficulties in purify-

ing them, fewer solutions were measured; so the results as a whole are probably less accurate than those obtained for the aliphatic amino acids.

The variation with temperature of the ionization constants of weak acids can be represented by equations of several types (3, 2, 4); that of Harned and Embree was found to fit these data, as shown by the comparison of observed  $pK$  and calculated  $pK$  values in Tables III and IV. This equation yielded values of  $\theta$ , the temperature of maximal ionization, and corresponding  $pK$  values for these temperatures,  $pK_{\max.}$ , as shown in Table V. Tables III and IV also include values for the changes in free energy, heat, and entropy calculated from the observed results by thermodynamic equations given in an earlier paper (8). Table V is a compilation of the values for  $\theta$ , the temperature of maximal ionization, and the corresponding values of  $pK_{\max.}$ . A more complete discussion of the results obtained with this and similar equations appears in another report.<sup>1</sup>

TABLE V  
*Maxima of First and Second Acid Ionization Constants and Temperatures of Maximal Ionization of Hydroxyamino Acids*

Amino acid	$pK_{1\max.}$	$\theta_1$	$pK_{2\max.}$	$\theta_2$
<i>dl</i> -Serine	2 128	58.6	7.568	153 0
<i>dl</i> -Threonine	2 054	54.0	7.600	147.5
<i>dl</i> -Allothreonine	2 087	44 0	7.469	152.6
<i>l</i> -Hydroxyproline	1.789	47.6	8 329	140.4
<i>l</i> -Proline	1 948	34.4	9 033	151.8

At comparable temperatures the values of  $pK_1$  for the hydroxyamino acids are 0.1 to 0.2 unit lower than the corresponding aliphatic amino acids, while the values of  $pK_2$  are 0.6 to 1.0 unit lower. The temperature of maximal ionization on the acid side is appreciably higher (11–21°) but on the alkaline side it is 5–11° lower.

#### SUMMARY

The thermodynamic ionization constants of *dl*-serine, *dl*-threonine, *dl*-allothreonine, *l*-hydroxyproline, and *l*-proline have been determined from 1–50° from electromotive force measurements of cells without liquid junctions. Values for the derived thermodynamic constants have been calculated.

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<sup>1</sup> Smith, P. K., and Smith, E. R. B., in preparation.

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# IMPROVEMENTS IN THE GASOMETRIC ESTIMATION OF CARBON MONOXIDE IN BLOOD

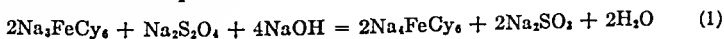
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A simple and accurate gasometric method for measuring the CO content of blood is useful for blood volume determinations, for the estimation of CO poisoning, and for checking the more speedy optical methods which, from time to time, have been devised for these and other purposes. In an attempt to remedy certain disadvantages in previous methods, Roughton (1) recently worked out a technique in which 2 cc. of the CO-containing blood are laked with 2 cc. of 1 per cent saponin, and then shaken in the dark for 2 to 3 minutes with 1.5 cc. of 2 per cent  $\text{Na}_2\text{S}_2\text{O}_4$  in 1 M glycinate buffer (pH 10). The  $\text{O}_2$  and  $\text{CO}_2$  of the blood are thereby chemically bound, whereas the  $\text{N}_2$  and physically dissolved CO of the blood are liberated into the gas phase; the CO bound to the Hb is liberated only to an insignificant extent. In the next stage of the analysis the CO of the COHb is liberated in full, without any other gas, by shaking for 10 minutes with 0.5 cc. of air-free 32 per cent  $\text{K}_3\text{FeCy}_6$ . The bound CO of the blood is thus easily measured without need of any special absorbent for CO. In this respect the method resembles that of Van Slyke and Neill (2) but eliminates the blank corrections of the latter. Tests given in the paper, over the range 0 to 30 per cent COHb, show an accuracy higher than that of the Van Slyke-Neill method, and indeed about equal to that of the more elaborate method of Sendroy and Liu (3). With one batch of  $\text{Na}_2\text{S}_2\text{O}_4$ , however, fallacious results were obtained. Since then, difficulties have been encountered with a few other samples of  $\text{Na}_2\text{S}_2\text{O}_4$  and of glycine. The investigations of these troubles, together with the extension of the method to concentrations of COHb higher than 30 per cent, have led us to modifications which are simpler, much quicker, and apparently more reliable than the original method of Roughton. Before the modifications are described, it will be useful to discuss briefly the difficulties just mentioned.

Part of the ferricyanide, which is added to liberate the CO from the COHb in the 10 minute shaking period, must clearly react with the excess of  $\text{Na}_2\text{S}_2\text{O}_4$  used previously for binding the  $\text{O}_2$  of the blood. This reaction probably follows the equation



Calculations show that, when only 0.5 cc. of 32 per cent ferricyanide is used, more than half of the latter may react in this way and hence be unavailable for liberating CO from the COHb. There is, however, no certainty that the oxidation-reduction reactions stop at the stage represented by Equation 1, especially if the hyposulfite, which is rarely better than 85 per cent pure, contains catalytic impurities. If, for example, the  $\text{Na}_2\text{SO}_3$  is further oxidized to  $\text{Na}_2\text{SO}_4$ , still more ferricyanide will be reduced and there will be serious danger of an inadequate amount being left over to liberate all the CO. An identical failure would occur either from some similar impurity in the glycine or if the ferricyanide deteriorated through contact with mercury, light, grease, or other agencies which cause it to darken in color. One or the other of these factors was probably responsible for the anomalous result reported with one batch of  $\text{Na}_2\text{S}_2\text{O}_4$  (1). In our recent work 1.0 cc. instead of 0.5 cc. of saturated ferricyanide has been used. The solution, prepared from analytical grade reagent, has after deaeration been stored in a burette with a rubber outlet and pinch-cock, rather than in contact with a greased stop-cock. The ferricyanide is best prepared fresh each day, and should be rejected if its color has darkened appreciably.

From Equation 1 it is also seen that a considerable amount of alkali is neutralized during the reaction between ferricyanide and  $\text{Na}_2\text{S}_2\text{O}_4$ . This raises a danger, not mentioned or met with in Roughton's paper, but since encountered with one sample of powdered glycine (origin not known); namely, that the pH, on shaking with ferricyanide, may not remain sufficiently alkaline to retain all the  $\text{CO}_2$ . In a blank experiment with water, in place of blood, there was with this sample of glycine a slow prolonged evolution of some gas when the water,  $\text{Na}_2\text{S}_2\text{O}_4$ -glycine, and ferricyanide were shaken for successive 5 minute periods, in the 50 cc. vacuous phase of the Van Slyke chamber.<sup>1</sup> On compression of the gas in the chamber to the 2 cc. mark, a pressure of several mm. was registered but on a further compression of the gas several times into the top of the chamber between the 0.5 cc. mark and the stop-cock the gas was all reabsorbed, as was also the case if air-free NaOH was added. This leaves little doubt that the gas is  $\text{CO}_2$ , the pH being low enough for the partial pressure of  $\text{CO}_2$  to reach a few tenths of a mm. when shaken at the 50 cc. mark; such a partial pressure would amplify to a few mm. on compression to the 2 cc. mark. It thus appears that, as regards alkalinity and buffer power, Roughton's method is too near the limit of safety, even though with the reagent brands used by him and recently by us (*viz.* Cenco  $\text{Na}_2\text{S}_2\text{O}_4$  and Paragon glycine) no trouble of this kind has arisen.

<sup>1</sup> Van Slyke, Dillon, and Margaria (4) report a similar evolution of gas from certain commercial samples of  $\text{Na}_2\text{S}_2\text{O}_4$  in alkali.

Two ways of eliminating these possible difficulties suggested themselves: Method A, to abandon the retention of  $\text{CO}_2$  by alkaline glycine buffer and instead to liberate most of this gas as well as the whole of the bound CO by shaking the blood-hyposulfite solution with ferricyanide at pH 6.0. The evolved  $\text{CO}_2$  is then absorbed with NaOH and the residual gas is solely CO; Method B, to replace the glycine buffer mixture by a much more alkaline  $\text{K}_3\text{PO}_4$ - $\text{K}_2\text{HPO}_4$  solution. Of these alternatives Method A is preferable, since at room temperature ( $20^\circ$ ) only 3 minutes shaking is needed to liberate all the CO, and the analysis is complete in 15 minutes instead of the 30 minutes taken by either Method B or by Roughton's method. The precipitate in the chamber is much less tenacious than that usually formed with Van Slyke's more acid ferricyanide reagent. Method B is, however, described, as we have used it extensively, and a slight modification of it is useful for the determination of the dissolved  $\text{N}_2$  content of blood (or other biological fluids). As one check on Methods A and B, we have used a modified and more accurate form of the Van Slyke-Neill method for CO in blood.

### *Description of Method A*

#### *Reagents Required—*

Caprylic alcohol.

1 per cent saponin.

2 per cent  $\text{Na}_2\text{S}_2\text{O}_4$  in saturated sodium borate. A stock of 4 per cent  $\text{Na}_2\text{B}_4\text{O}_7 \cdot 2\text{H}_2\text{O}$  (analytical grade) is made up. At the beginning of the day, 50 cc. of this are placed in a 50 cc. Erlenmeyer flask, 1 gm. of solid  $\text{Na}_2\text{S}_2\text{O}_4$  dropped in, and the flask quickly corked and shaken with only a minute bubble of air therein. The solution is at once transferred to a 50 cc. burette and stored under oil.

Air-free 32 per cent  $\text{K}_3\text{FeCy}_6$  (analytical grade). This is freshly prepared by shaking in a vacuum tonometer and transferring to a burette with a rubber outlet and pinch-cock. If it darkens, a fresh supply must be made.

Air-free phosphate buffer. Dissolve 13.6 gm. of  $\text{KH}_2\text{PO}_4$  and 3.5 gm. of  $\text{K}_2\text{HPO}_4$  (both analytical grade) in water and dilute to 100 cc. Deaerate and store in the ordinary way. The potassium salts are used because of their high solubility.

Air-free 10 per cent NaOH. This need not be carbonate-free.

*Procedure*—4 drops of caprylic alcohol are drawn into the Van Slyke chamber and 2 cc. of 1 per cent saponin are placed in the cup. 1 to 2 cc. of blood (according to whether the per cent COHb is greater or less than 50) are drawn directly into the chamber, followed by the 2 cc. of saponin.

After 1 minute for completion of laking, 2 cc. of the 2 per cent hyposulfite-borate solution are placed in the cup and the lower 1.5 cc. drawn into the chamber. The mercury is lowered to the 50 cc. mark, the chamber covered with black paper, and the solution shaken at the usual rate for 2 minutes. The evolved gases are quantitatively ejected and 1.5 cc. of deaerated 32 per cent  $K_3FeCy_6$  placed in the cup. The lower 1.0 cc. of this is drawn into the chamber and the remaining 0.5 cc. discarded. 1.0 cc. of the deaerated phosphate buffer is similarly introduced into this chamber, the tap sealed, the mercury lowered to 1 cm. below the 50 cc. mark, and the mixture shaken for a total of 3 minutes. Three times during the shaking the motor is temporarily stopped, and the mercury raised to the 50 cc. mark, so as to mix the solution in the stem of the chamber with the main body of the solution. In this way destruction of the ferricyanide by the mercury is minimized.

The gas is then compressed nearly to atmospheric pressure and 2.0 cc. of the air-free NaOH placed in the cup. 1 cc. of the latter is run into the chamber, 15 to 30 seconds being allowed for  $CO_2$  absorption to be completed. Finally, only a small bubble is left in the top of the chamber. The solution is then lowered to the 2.0 cc. mark and after 1 minute for drainage the pressure,  $p_1$ , read. The gas is then quantitatively ejected and the pressure read again,  $p_2$ . The CO content in volumes per cent then equals  $(p_1 - p_2 - c) \times \text{constant}$ .

The  $c$  correction (usually about 0.5 mm.) is determined in the usual way with water in place of blood, and the constant is obtained from the appropriate column in Table 30 of Peters and Van Slyke (5). Thus if the blood sample equals 1 cc.,  $p_1 = 134.2$  mm.,  $p_2 = 47.8$  mm.,  $c = 0.5$  mm., and the temperature =  $21.6^\circ$ .

$$\text{Volumes per cent CO} = 85.9 \times 0.2445 = 21.003.$$

The precipitate of methemoglobin remaining in the chamber is readily removed by shaking with dilute alkaline hyposulfite.

*Notes on Preliminary 2 Minute Shaking*—A control experiment showed that 2 minutes shaking was long enough to extract the dissolved  $N_2$  of the blood and reagents. Roughton calculated that the dissociation of COHb which could occur during this period was negligible if the per cent COHb lay between 0 and 30. With blood samples containing up to 100 per cent COHb a direct test is preferable to a calculation. In three experiments, in which 1 cc. of blood 100 per cent saturated with CO was shaken in the darkened chamber for 16 minutes with the saponin-hyposulfite mixture, we found an average rate of CO evolution of 0.00024 cc. of CO per minute; this would only cause a negligible error of 1 part in 500 in the CO determination.



### Description of Method B

**Reagents**—These are the same as in Method A save that the 10 per cent NaOH is not required and the acid phosphate buffer is replaced by air-free alkaline phosphate buffer consisting of 3.5 gm. of  $K_2HPO_4$  and 17.1 gm. of  $K_3PO_4$  dissolved in water and diluted to a total volume of 100 cc.

**Procedure**—This is exactly as in Method A save that (a) the second shaking is continued for 10 minutes, instead of 3 minutes, and a check reading is taken after 5 minutes further shaking to make sure that the gas evolution is complete, and (b) no absorption by NaOH is required, the pressure reading  $p_1$  at the 2.0 cc. mark being taken directly after the shaking is finished.  $p_2$  is the pressure reading after the ejection of all gas.

Volumes per cent CO =  $(p_1 - p_2 - c) \times \text{constant}$  given by Peters and Van Slyke.

**Determination of Dissolved  $N_2$  of Blood**—This is readily done by a slight variation of Method B. 2 cc. of 1 per cent saponin, 1.5 cc. of the hyposulfite-borate solution, and 1.0 cc. of the alkaline phosphate buffer are evacuated and shaken for 2 minutes in the Van Slyke chamber. The evolved gas is quantitatively ejected and 2 cc. of the blood are then drawn into the chamber and the mixture shaken *in vacuo* in the dark for 2 minutes. The pressure reading  $p_1$  at the 2.0 cc. mark is taken, the  $N_2$  evolved from the blood is quantitatively ejected, and the pressure reading  $p_2$  read.

Volumes per cent dissolved  $N_2$  then equals  $(p_1 - p_2) \times \text{constant}$  as before.

**Modification of Van Slyke-Neill Method**—In the original method all the gases of the blood are evolved by shaking *in vacuo* with acid ferricyanide; the  $CO_2$  and  $O_2$  are then absorbed by alkaline hyposulfite and the pressure of the residual gases, CO and  $N_2$ , measured. The volume per cent CO is thence obtained by subtracting the dissolved  $N_2$  content of the blood, this being calculated from the solubility coefficient of  $N_2$  in blood and the pressure of  $N_2$  with which the blood is supposedly in equilibrium at the time of the drawing. A blank correction is also made for the slight reabsorption of CO by the reduced hemoglobin formed when the hyposulfite absorbent is added.

We have increased the accuracy of the method by determining the actual  $N_2$  content of the blood in a duplicate sample (as just described) instead of by calculation, and by replacing the hyposulfite absorbent by a freshly prepared air-free solution of 5 per cent pyrogallol in 10 per cent NaOH.

The latter reagent, unlike hyposulfite, does not absorb any CO, as is shown by the fact that its use in Method A in place of the ordinary 10 per cent NaOH leads to identical values of the CO content. A possible disadvantage of the reagent is that small amounts of CO may be liberated from it, especially when the  $pO_2$  is high and prolonged shaking is required for

absorption to be complete, as in the Haldane gas analysis apparatus. In the Van Slyke apparatus the absorption is complete in 1 minute if 1 cc. of pyrogallol is used and the gas is kept above the 2 cc. mark throughout the absorption. Under these circumstances the  $O_2$  pressure is small and the time of contact of each pyrogallol molecule with the gas phase so short that no appreciable error from evolved CO arises, as is shown by several controls (e.g., the  $O_2$  capacity of blood as measured with the ordinary hyposulfite absorbing reagent agrees with that obtained with the pyrogallol reagent). 1 cc. of this pyrogallol solution is used, and about 1 minute is allowed for its admission from the cup into the Van Slyke chamber. The gas in the chamber is kept just a little below atmospheric pressure during the absorption.

TABLE I  
*Estimations of Volumes Per Cent CO in Blood by Various Methods*

Experiment No.	Mixture	Method A	Aerated blood			Van Slyke-Neill method (modified)	Method B	Glycine*
			CO content	$O_2$ capacity	Sum			
1	4.49 (1)	4.43 (1)						
	6.05 (1)	6.06 (2)						
	7.59 (1)	7.54 (2)						
	10.25 (1)	10.24 (1)						
2		20.49 (2)	0.41 (1)	20.10 (1)	20.51 (1)			
3		21.24 (2)	0.54 (2)	20.63 (2)	21.17 (2)			
4			0.36 (1)	21.61 (2)	21.97 (1)		22.03 (2)	
5		18.81 (2)				18.74 (1)		
6		20.94 (2)				20.86 (2)		
7		3.20 (3)				3.19 (3)		3.24 (2)
8		21.81 (2)					21.77 (3)	21.84 (2)
9							20.42 (3)	20.43 (3)

The figures in parentheses denote the number of determinations.

\* 1.0 cc. of 32 per cent  $K_2FeCy_6$  was used in place of 0.5 cc.

*Checks of Methods*—In a total of twenty analyses on human blood the average discrepancy between duplicate determinations was found to be 0.055 volume per cent CO both in the case of Method A and Method B, which with 1 cc. blood samples corresponds to a CO pressure of 0.2 mm. of Hg on the Van Slyke manometer. The maximum precision of the Van Slyke technique is thus reached. In only two out of the twenty determinations was a discrepancy greater than 0.10 volume per cent found, the highest being 0.14.

Table I summarizes the various checks we have applied.

Experiment 1 shows that the values by Method A check very closely with those of calculated mixtures of aerated blood and blood 100 per cent saturated with CO by shaking with a gas mixture containing CO.

Experiments 2, 3, and 4 show that the CO capacity of blood by Methods A and B agrees with the O<sub>2</sub> capacity of the same blood (as measured by the ordinary Van Slyke-Neill O<sub>2</sub> procedure) if there be added to it the small CO content of the aerated blood, which in the case of our present subjects amounts to about 0.4 volume per cent.

Experiments 5, 6, and 7 show the comparison of Method A with the Van Slyke-Neill method (modified). In Experiment 5, the blood was equilibrated with a CO-O<sub>2</sub> mixture and so no allowance for dissolved N<sub>2</sub> was needed.

Experiments 7, 8, and 9 show the mutual agreement between Methods A and B and the glycine method (if 1.0 cc. of ferricyanide is used in the latter and all the reagents are carefully controlled). Experiment 7 also shows the agreement between Method A and the Van Slyke-Neill and glycine methods.

The success of this series of tests gives confidence that the principles and technique of the methods are reliable and reproducible.

*Avoidance of Hyposulfite in Method A*—No trouble has been found in this method with Cenco brand hyposulfite, but it is possible that other brands of Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> might occasionally lead to false results. At the end of the research we made several tests to see whether the hyposulfite could be omitted entirely from the borate solution used for the preliminary shaking and ejection of dissolved N<sub>2</sub>. In this case some O<sub>2</sub> would be liberated as well as N<sub>2</sub> during the preliminary shaking but it was found by calculation from the hemoglobin dissociation curves that no appreciable liberation of CO from COHb should be caused thereby. This was confirmed by three comparisons of the CO content of blood solution as estimated by (1) Method A unmodified, and (2) Method A modified by omission of hyposulfite from the borate solution and with 1.5 cc. of air-free 5 per cent pyrogallol in 10 per cent NaOH in place of the usual 10 per cent NaOH absorbent. In the modified Method A, some of the O<sub>2</sub>, most of the CO<sub>2</sub>, and the whole of the CO of the blood are liberated by the shaking with ferricyanide. The alkaline pyrogallol then absorbs the O<sub>2</sub> and CO<sub>2</sub>, leaving the residual gas, which is pure CO, for direct manometric measurement. The results of the comparisons were as follows:

Blood sample No. . . . .	I	II	III
Method A, vol. % CO . . . .	0 29	6 06	11 78
" " without Na <sub>2</sub> S <sub>2</sub> O <sub>4</sub> , vol. % CO	0 30	6 07	11 81

Whether the blood contains much O<sub>2</sub> as in Sample I or but little as in Sample III, agreement within experimental error is obtained. The hyposulfite may therefore safely be omitted, but the borate should be retained,

so as to keep the pH alkaline enough during the first 2 minutes shaking for the rate of dissociation of COHb to be negligible.

#### DISCUSSION

The improved methods described above apparently reach the maximum accuracy attainable in the Van Slyke apparatus. The best of these, *viz.* Method A, is as easy and as quick as that of the Van Slyke-Neill determination of the O<sub>2</sub> content of blood. No further search for simplicity or speed of working therefore seems called for.

Methods A and B and that of Roughton differ from previous Van Slyke methods in giving only the chemically combined CO of the blood and not, in addition, the dissolved CO, since the latter is eliminated during the preliminary 2 minute shaking period. This is an advantage in determinations of the total hemoglobin of the blood by CO capacity methods, since the blank correction for dissolved CO is thereby eliminated. Any foreign gases which might be present in physical solution in the blood (as in illuminating gas poisoning) would also be removed during the 2 minute shaking, whereas in the Van Slyke-Neill method they would add to (and confuse) the blank correction for dissolved N<sub>2</sub> and in the Sendroy-Liu method they might in some cases also be absorbed by the cuprous chloride reagent used for absorbing CO. The present methods are thus perhaps more specific than previous gasometric methods.

An advantage of Roughton's method was that it could be satisfactorily used not only in the Van Slyke, but also in the Barcroft-Warburg apparatus. The same applies to Method B, the Barcroft procedure for which is the same as used by Roughton for his glycine method. If the usual provision be made for absorbing evolved CO<sub>2</sub> (6), there seems no reason why Method A also should not work in the Barcroft apparatus. Owing to lack of immediate facilities, this has not yet been tested.

The CO contents of the blood of the normal subjects used in Table I run somewhat higher than usual; we have had other cases running up to over 1 volume per cent. We hope to investigate this further, with reference not only to smoking but also to the claim of the French investigators (7) that CO is formed endogenously in the body, especially during deficient carbohydrate metabolism.

#### SUMMARY

Modifications have been made in Roughton's recent method for estimating CO in blood with the Van Slyke apparatus; the main object was to avoid difficulties occasionally encountered with certain brands of reagents. Several new methods are described; of these, the most serviceable is as speedy and simple as the ordinary Van Slyke-Neill determination of

the oxygen content of blood, and has, according to numerous tests, a precision equal to the highest that can be attained with the Van Slyke technique.

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# TYROSINASE AND PHENOLIC PRESSOR AMINES

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Recent studies of the mechanisms involved in the production of experimental renal hypertension show that humoral pressor agents are involved. It appears that either one or both of two types of pressor substance may be liberated from the kidney itself, but that a single type of substance acts to produce the peripheral vasoconstriction. One type of substance that may be liberated from the kidney has the properties of a non-dialyzable protein, like the renin of Tigerstedt and Bergmann (1). This substance reacts with certain serum constituents to produce a dialyzable amine type of pressor substance, according to Page, Koehlstaedt, and Helmer (2, 3), who called this substance angiotonin, and according to Braun-Menendez and coworkers (4), who called it hypertensin. Another type of substance that may be liberated from the kidney has the properties of a dialyzable pressor amine that is very possibly tyramine or hydroxytyramine, as developed by the work of Holtz (5, 6) and of Bing (7, 8) and their coworkers. Whatever the substances liberated by the kidney may be, it appears that the humoral agent producing a peripheral vasoconstriction is an amine in type, and very possibly a phenolic amine.

The pressor effect of renin can be inactivated by tyrosinase preparations in the presence of oxygen, and the product of its interaction with serum, angiotonin or hypertensin, can be similarly inactivated, as shown by Schroeder and Adams (9) and confirmed by Croxatto and Croxatto (10). The latter workers also showed that hypertensin could be readily inactivated by amine oxidase preparations in the presence of oxygen. Schroeder and Adams studied the effects of injected tyrosinase preparations on the blood pressures of animals with experimental renal hypertension, and also of persons having arterial hypertension (11). Falls in blood pressure and other signs of clinical improvement were obtained, and the conclusion was drawn that it was probable that some phenolic substance acting as a humoral agent was altered. A recent note by Schroeder (12) reports that the injection of amine oxidase preparations lowered the blood pressure of animals with experimental renal hypertension.

The tissues of mammals, as studied by Bhagvat and Richter (13), do not normally contain any considerable amounts of tyrosinase or other phenoloxidases. The physiological inactivation of phenolic amines in

man appears to be largely carried out by some esterifying mechanism, as shown by Richter and Richter and MacIntosh (14, 15). It is therefore of considerable interest to value the kinetics of phenoloxidase-catalyzed oxidations of a number of phenolic pressor amines, under approximate physiological conditions, in order to estimate the rôle any normally occurring amounts could play in the inactivation of such compounds in the body. Such data also furnish bases for consideration of the possible effects of injected amounts of tyrosinase or other phenoloxidase preparations.

Basic to the consideration of the action of tyrosinase on the oxidation of phenolic pressor amines are the observations of Keilin and Mann (16) and of Nelson and his coworkers (17-19) that show that different preparations may vary considerably in their relative actions on monophenols and *o*-diphenols. Both of these types of activity appear to belong to the same enzyme complex, as they bear a proportionality to the same copper content. However, since the activities vary with the purity and method of purification, each enzyme preparation must be defined in terms of both monophenolase and *o*-diphenolase activities. This was done in the present studies, and modifications of previously described preparative methods were required to retain a reasonable proportioning of such activities in purified preparations.

### *Purified Tyrosinase Preparations*

The methods described by Nelson and coworkers (17-19) were first tried, but met with little success, owing to rapid inactivation and processing losses. The methods of Keilin and Mann (16) were then tried, and after some modification it was possible to obtain quite good yields of stable preparations which were 30 to 40 times more active per unit weight than crude aqueous extracts. In our experience, only a white cultivated variety of the common mushroom gave good results, a spotted brown and white variety gave fair results, while a brown variety gave precipitates that were difficult to handle and high inactivation and processing losses occurred.

The tyrosinase activity of the preparations was valued by methods similar to those of Adams and Nelson (17), but modified by use of available equipment. The assays were made with an Aminco Warburg apparatus, with seven 15 ml. vessels shaken at 120 oscillations per minute in a 30.0° water bath. In the body of the flasks were placed 1.0 ml. of 0.4 M sodium phosphate buffer, pH 7.0, containing 2.5 mg. of gelatin, and 0.5 ml. of enzyme preparation at a dilution to cause an oxygen uptake of about 5 microliters per minute. In the side arm was placed either 0.5 ml. of a solution containing 0.1 mg. of catechol and 5 mg. of hydroquinone per ml. for catecholase activity determination or 0.5 ml. of 0.04 M *p*-cresol for cresolase activity determination. The solutions were attemperated for



15 minutes with shaking at a 2 cm. stroke, and then mixed and shaking continued at a 4 cm. stroke. For catecholase activity, readings were made at 2 minutes, while for cresolase activity, readings were made at 5 minute intervals. In either case, a unit (catecholase = Ca. u., cresolase = Cr. u.) is defined as that which causes an uptake of 10 microliters of  $O_2$  per minute under the conditions used. The unit was found to be seven-twelfths that of the unit similarly defined by Adams and Nelson for their equipment.

*Crude Extract*—5 pounds of fresh mushrooms were ground twice, mixed with 200 ml. of water, and then pressed in a canvas bag. Regrinding with sand and 400 ml. of water, repressing, then another grinding with sand and 600 ml. of water gave about 2700 ml. of reddish brown liquid.

Non-dialyzable solids, 6.52 mg. per ml., 5 Ca. u. per mg., 7 Cr. u. per mg. Total Ca. u., 92,000; total Cr. u., 123,000.

*Ammonium Sulfate Precipitation, Followed by Dialysis*—Crude extract adjusted with N acetic acid to pH 5 was treated with 700 gm. per liter of ammonium sulfate and the pH adjusted to 4.8, with stirring for 2 hours. Centrifugation in a Sharples machine at 25,000 R.P.M. completed the solid separation in an hour, while filtration attempts were discouraging. The solid was resuspended in 450 ml. of water with stirring for 0.5 hour, and the mixture then dialyzed within 36/32 Visking cellophane tubing by shaking in running tap water for 24 to 40 hours. Insoluble material was centrifuged out and the centrifugate made up to 1000 ml.

Non-dialyzable solids, 4.09 mg. per ml., 34 Ca. u. per mg., 29 Cr. u. per mg. Total Ca. u., 139,000;<sup>1</sup> total Cr. u., 118,000.

*Lead Acetate Treatment*—Aliquots of 20 ml. were taken and treated with 0.6 to 1.4 ml. of 0.05 saturated lead acetate solution to determine the approximate amount required to effect removal of color to a green filter reading of about 300 on a Klett colorimeter. The total batch had to be treated with a 20 to 25 per cent greater ratio of the lead acetate solution to obtain equivalent decolorization, and was then centrifuged.

Non-dialyzable solids, 1.47 mg. per ml., 57 Ca. u. per mg., 41 Cr. u. per mg. Total Ca. u., 83,000; total Cr. u., 59,000.

*Clarification with Calcium Phosphate*—Treatment of the extract with some freshly precipitated calcium phosphate at pH 7.0, by stirring for 0.5 hour

<sup>1</sup> Assay shows an increase in total Ca. u. during ammonium sulfate precipitation followed by dialysis, and this was shown by several, but not all, the preparations made. Keilin and Mann (16) also observed such an increase and postulated the presence of a proenzyme that was activated during dialysis.

and then allowing it to stand overnight, gave a clear solution after centrifugation, about two-thirds as colored as after the lead acetate treatment.

Non-dialyzable solids, 1.07 mg. per ml., 76 Ca. u. per mg., 55 Cr. u. per mg. Total Ca. u., 80,000; total Cr. u., 58,000.

The calcium phosphate was prepared by mixing equal volumes of 0.15 M calcium acetate and 0.1 M disodium phosphate and adjusting the pH to that at which the suspension was to be used. 1 ml. of this is equivalent to 15 mg. of tricalcium phosphate.

*Calcium Phosphate Adsorption and Elution*—80 per cent of the activity was adsorbed by adjustment of the pH to 6.6 and treatment with 1 gm. of calcium triphosphate in suspension at pH 6.6 for each 20,000 Ca. u. by stirring for 0.5 hour. The adsorption precipitate was centrifuged off with the Sharples machine, and then stirred for an hour into a suspension with 75 ml. of 0.5 M potassium phosphate buffer at pH 7.5. The eluate was centrifuged off and elution repeated twice. The combined eluates were dialyzed against running tap water for 40 hours.

Non-dialyzable solids, 1.41 mg. per ml., 140 Ca. u. per mg., 90 Cr. u. per mg. Total Ca. u., 51,000; total Cr. u., 32,000.

By treatment of such solutions with small amounts of 0.01 saturated lead acetate and with calcium phosphate suspensions, about half of the enzymic activity could be obtained in preparations showing about 210 Ca. u. per mg. with 110 Cr. u. per mg.

For use in experimental renal hypertension in animals, or in arterial hypertension in man, more concentrated solutions of the calcium phosphate eluate preparations were desired. These were prepared by precipitating relatively large volumes of eluate preparations with 700 gm. per liter of ammonium sulfate and adjusting the pH to 4.8. The precipitate was taken up with 0.9 per cent sodium chloride solution at pH 7.0, and made 1:50,000 in phenylmercuric acetate for preservation. Such solutions contained non-dialyzable solids, 3.72 mg. per ml., 300 Ca. u. per mg., 90 Cr. u. per mg. The stability of the more highly purified preparations, when kept in the refrigerator at about 5°, was very satisfactory. One preparation that was tested almost weekly for 5 months showed no change in either catecholase or cresolase activity. Another preparation carefully restandardized after 3 months showed no change in either type of activity.

These tyrosinase preparations could be rapidly inactivated by heating above 60°, with the attendant precipitation of coagulated proteins. Study of this heat inactivation showed that by keeping the preparations at 60° for 40 minutes, 95 per cent or more of the catecholase and cresolase activity was destroyed. Such heating did cause precipitation of some material which almost completely dissolved on cooling and shaking.

*Tyrosinase Oxidations of Phenolic Amines*

The relative activity of tyrosinase preparations when tested upon monophenols and *o*-diphenols may not be reflected in their relative activity when tested upon monophenolic and *o*-diphenolic amines. This question was especially studied, since it appeared possible that, although preparations might vary considerably in their relative catecholase to cresolase activity, certain generalizations might be made for the action of tyrosinase on types

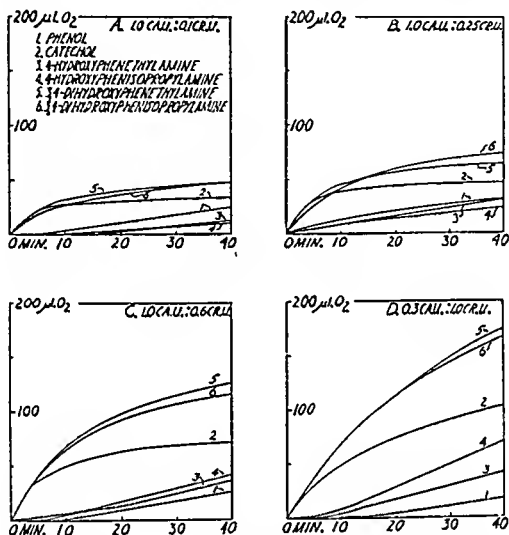


FIG. 1. Oxygen uptake in microliters against time in minutes for preparations having different ratios of catecholase to cresolase activity. A and B may be considered high catecholase, C and D high cresolase preparations. The experiments were carried out at  $30^\circ$  with  $0.01\text{ M}$  amine hydrochloride substrates in  $0.2\text{ M}$  sodium phosphate buffer, pH 7.0, and  $0.5\text{ ml}$ . of enzyme of the concentration shown per  $\text{ml}$ . used in a  $2\text{ ml}$ . total volume. The curve numbers in all four sections correspond to the substrates listed in A.

of phenolic amines, and particularly on phenolic pressor amines that might occur in the body under physiological conditions. Confirmation of this idea was indeed obtained by the study of four enzyme preparations, varying widely in their relative catecholase to cresolase activity upon the same series of six amines. Three of these amines were monophenolic; the other three were diphenolic and derivatives of catechol.

Three enzyme preparations were made by following the previously outlined methods, and represented a range from a relatively high catecholase to cresolase ratio of 10:1 through a ratio of 10:2.5, and finally of 10:6.

The oxidation rates were studied for these three preparations at 30°, with 0.5 ml. of enzyme preparation containing 1.0 Ca. u. per ml. and attendant cresolase unitage, and 0.5 ml. of 0.04 M phenolic amine, in a total volume of 2.0 ml. Curves showing the results are given as A, B, and C of Fig. 1. A high cresolase preparation was also made specially for this comparative study by fractional ammonium sulfate precipitation of a crude extract of brown mushrooms. This preparation showed a catecholase to cresolase ratio of 10:33, and 0.5 ml. of enzyme preparation containing 1.0 Cr. u. per ml. and but 0.3 Ca. u. per ml. was used for the studies shown in Fig. 1, D.

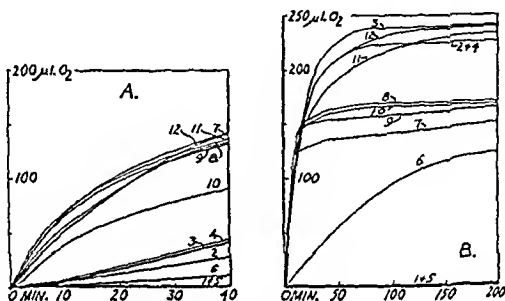


FIG. 2. Oxygen uptake in microliters against time in minutes, at 30° with 0.01 M amine hydrochloride substrates in 0.2 M sodium phosphate buffer, pH 7.0, and 0.5 ml. of enzyme used in a 2 ml. total volume. In A the enzyme solution used contained 1.0 Ca. u. and 0.6 Cr. u. per ml., and in B the enzyme concentration was 24 times greater. Curve 1, 3-hydroxyphenethylamine; Curve 2, 4-hydroxyphenethylamine; Curve 3, 4-hydroxyphenethylmethanamine; Curve 4, 4-hydroxyphenisopropylamine; Curve 5, 3-hydroxyphenethanolmethanamine; Curve 6, 4-hydroxyphenethanolmethanamine; Curve 7, 3,4-dihydroxyphenethylamine; Curve 8, 3,4-dihydroxyphenethylmethanamine; Curve 9, 3,4-dihydroxyphenisopropylamine; Curve 10, 3,4-dihydroxyphenethanolamine; Curve 11, *dl*-3,4-dihydroxyphenethanolmethanamine; Curve 12, *l*-3,4-dihydroxyphenethanolmethanamine.

The results from monophenolic and diphenolic amine substrates fall into separate groups with each of the several enzyme preparations. The principal point of difference in the action of the four preparations is the greater spread of the curves with increased cresolase activity. The cresolase activity appears to potentiate the catecholase activity of the preparations of high cresolase activity with respect to all six of the substrates studied.

To further the idea that oxidation behavior of phenolic pressor amines may be classified on the basis of the number and position of the benzene ring substituents, additional studies were carried out on a rather varied series of phenolic and *o*-diphenolic amines. For the initial study, a single

tyrosinase preparation with a catecholase to cresolase ratio of 10:6 was used, with enzyme and substrate concentrations as in the studies of A, B, and C of Fig. 1. A second study was carried out with enzyme concentrations 24 times as great and with longer periods of observation, to indicate the total extent of oxidation of these compounds by tyrosinase. The results of these studies are shown in Fig. 2, and it should be noted that here again the *initial* rates of the effects upon monophenolic and diphenolic amines are separable into groups, with the exception that the 3-hydroxy-phenylalkyl- (or alkanol-) amines are not oxidized at all. Another point of interest is that the ethanolamine side chain appears to inhibit oxidation in both series, though not notably with adrenalin.

The total extent of tyrosinase oxidation of these various substrates, as shown by Fig. 2, B, indicates that the course of the oxidation, aside from phenolic oxidation, may differ greatly among the various compounds. This is perhaps related to the findings of Beyer (20), using certain phenolic pressor amines and crude potato tyrosinase preparations, that total oxidation may involve the splitting of ammonia from the molecule, as well as the production of indole derivatives and melanins, as found by Duliere and Raper (21).

#### *Dissociation Constants of Tyrosinase and Phenolic Pressor Amines*

The kinetic dissociation constants of tyrosinase with some typical phenolic pressor amine substrates were determined. These substrates were chosen because of their physiological or pharmacological interest. Although calculations were planned to be made that would apply to body temperatures, the studies were carried out in the Warburg apparatus at 25°. This was done in order to obtain sufficient experimental precision, since the rates determined must be those during the first few minutes of oxidation, and differences between bath and room temperatures of as much as 5° caused manometer shifts affecting the reading for the 1st minute owing to the removal of the vessels from the bath for mixing. Substrate concentrations varied from 0.002 to 0.02 molal. Enzyme concentrations were 3 Ca. u. per ml. with 1.8 Cr. u. per ml. for the monophenolic substrates, and 0.6 Ca. u. per ml. with 0.36 Cr. u. per ml. for the diphenolic substrates. Determinations were repeated in most cases at one-half these enzyme concentrations, and checks were obtained. For the monophenolic amines, the rates used were the maximum rates obtained over a 5 minute period after the initial lag period had ceased, and usually occurred between 8 and 13 minutes, often continuing constant for an additional 5 minutes. For the diphenolic amines, the oxidation rates of which rapidly decrease with time, the rates chosen were the maximum rates observed over a 1 minute interval, usually falling within the 1st or 2nd minute interval. By plotting  $1/V$ , where  $V$  = microliters of  $O_2$  uptake per minute, against

$1/S$ , where  $S$  = molal substrate concentration, the ordinate intercept  $1/V_{\max}$ , and the slope of the straight line  $K_s/V_{\max}$ , were determined, as suggested by Lineweaver and Burk (22).  $K_s$ , the dissociation constant of the intermediate enzyme-substrate compound, is then easily calculable.

#### DISCUSSION

The data presented with regard to the interaction of oxygen and the phenolic pressor amines in the presence of tyrosinase permit some calculations to be made with respect to the likelihood that tyrosinase plays any physiological rôle in the inactivation of these compounds in the body. Further, such calculations can indicate the order of magnitude of the amounts of tyrosinase that would have to be introduced into the body from the outside to exert an effect comparable to normal rates of destruction of these phenolic pressor amines.

By way of example, consider the oxidation rate of tyramine in a concentration of  $10^{-5}$  molal with an enzyme concentration of 3.0 Ca. u. and 1.8 Cr. u. per ml. by substitution of the data given in Table I for these conditions into the rate relation  $1/V = K_s/V_{\max} (S) + 1/V_{\max}$ . Such substitution gives  $1/V$  to be about 24, or  $V$  to be about 0.04 microliter of  $O_2$  per minute, or about  $0.02 \times 10^{-7}$  mole of  $O_2$  per minute. On the basis that physiological inactivation would occur with an uptake of 1 mole of  $O_2$  per mole,  $10^{-6}$  mole of tyramine under these conditions would require about 500 minutes to be inactivated with an enzyme concentration of 3.0 Ca. u. and 1.8 Cr. u. per ml. Such conditions of amounts and concentrations of tyramine would be approximated in the blood stream of an experimental animal immediately following the intravenous injection of  $10^{-6}$  mole per kilo (0.18 mg. per kilo of hydrochloride) of tyramine. The studies of Clark and Raventos (23) on the relationship between dosage and duration of physiological actions of tyramine in cats and in man showed that a dosage of  $10^{-6}$  mole of tyramine per kilo is inactivated in less than 20 minutes.

A similar calculation made from the data obtained on *l*-adrenalin in a concentration of  $10^{-7}$  molal with an enzyme concentration of 0.6 Ca. u. and 0.36 Cr. u. per ml. would give  $V$  to be about  $2 \times 10^{-4}$  microliter of  $O_2$  per minute, or about  $10^{-11}$  mole of  $O_2$  per minute. On the basis that physiological inactivation would occur with an uptake of 0.5 mole of  $O_2$  per mole,  $10^{-8}$  mole of adrenalin under these conditions would require about 500 minutes to be inactivated with the enzyme concentration of 0.6 Ca. u. and 0.36 Cr. u. per ml. Such conditions of amounts and concentrations of adrenalin would be approximated in the blood stream following the intravenous injection of  $10^{-8}$  mole per kilo (0.0018 mg. per kilo) of adrenalin. The duration of pressor and other responses in cats from such a dosage is less than 5 minutes, and is most commonly 2 to 3 minutes.

Certainly, no such considerable amounts of tyrosinase as 3.0 or even 0.6 Ca. u. per ml. exist normally in the blood or tissues of animals yet studied, as demonstrated by Bhagvat and Richter (13). If a tyrosinase-catalyzed oxidation were the principal oxidative mechanism, 25 to 100 or more times these concentrations of enzyme would be required. Although tyrosinase is thus indicated as being an unimportant mechanism normally, it might be suggested that its injection could be resorted to in order to increase the capacity of the body to inactivate phenolic pressor amines. So far as such compounds are represented by tyramine and adrenalin, amounts of tyrosinase approximating 25 to 100 or more times 3.0 or even 0.6 Ca. u. per ml. of body fluid would be required to be injected to approach de-

TABLE I  
*Tyrosinase Oxidations of Phenolic Amines*

The experiments were carried out at 25° in 0.2 M sodium phosphate buffer, pH 7.0, with substrate concentrations of from 0.002 to 0.02 molar

Phenolic amine	Enzyme concentration		$\frac{1}{V_{\max}}$	$\frac{K_s}{V_{\max}}$	$K_s$
	Ca. u. per ml	Cr. u. per ml		$\times 10^4$	
4-Hydroxyphenethylamine (tyramine)	3.0	1.8	0.104	2.42	0.0024
	1.5	0.9	0.206	4.03	0.0020
4-Hydroxyphenisopropylamine (paredrine)	3.0	1.8	0.051	3.44	0.0067
	1.5	0.9	0.100	6.00	0.0060
3,4-Dihydroxyphenethylamine (hydroxytyramine)	0.6	0.36	0.040	2.00	0.0050
	0.3	0.18	0.076	4.02	0.0053
3,4-Dihydroxyphenisopropylamine (hydroxyparedrine)	0.6	0.36	0.047	1.54	0.0033
	0.3	0.18	0.085	2.90	0.0034
dl-3,4-Dihydroxyphenethanolmeth- ylamine (dl-adrenalin)	0.6	0.36	0.045	3.24	0.0072
	0.3	0.18	0.090	6.80	0.0075
l-3,4-Dihydroxyphenethanolmeth- ylamine (l-adrenalin)	0.6	0.36	0.035	4.60	0.0131

struction rates comparable to those normally possessed by the body. These amounts are very large and are in excess of those that were used by Schroeder and Adams (9, 11) for the therapy of hypertension in animals and man, and it would seem probable that explanations other than increased destruction of phenolic pressor amines are required for the effects they reported.

#### SUMMARY

1. Methods were developed for the purification of extracts of common white mushrooms, giving good yields of stable tyrosinase preparations.
2. The ratio of catecholase to cresolase activities of such preparations was not greatly altered in the course of such purification.

3. The initial oxidation rates of diphenolic amines that were derivatives of catechol were greater than those of monophenolic amines with tyrosinase preparations, whether they are high or low in relative catecholase to cresolase activity.

4. Tyrosinase preparations oxidize a large number of monophenolic and o-diphenolic pressor amines, with the exception of those substituted with a single hydroxyl group in the 3 position relative to the side chain.

5. The extent of total oxidation of a number of phenolic pressor amines is quite variable, probably due to different pathways of the oxidation of the side chain with its amino group.

6. The kinetic enzyme dissociation constants of tyrosinase-amine combinations were found to be of the same order for the monophenolic and diphenolic amine types studied.

7. The dissociation constants and the oxidation rates of tyrosinase-amine combinations are such that this oxidation mechanism cannot account for any considerable part of the inactivation of tyramine or epinephrine in normal animals, and such oxidation is not more probable for related phenolic pressor amines.

8. Injections of large amounts of highly active tyrosinase preparations into animals would be required to produce rates of oxidation comparable to normal inactivation rates.

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# HEMICELLULOSES AND PECTIC MATERIALS FROM COTTON-WOOD, *POPULUS MACDOUGALI*

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Studies on hemicelluloses of wood extending back more than half a century enable us to draw some general conclusions as to their composition and structure. The size of the molecules as they exist in the cell wall, the manner in which they are combined with other cell wall constituents, and their origin are still matters of conjecture. However, enough is known about these substances and other closely related materials for us to draw some conclusions on these points also.

Polyuronide hemicelluloses from hardwoods are usually composed of a monomethoxyuronic acid combined with a series of molecules of *d*-xylose (1). The acid is probably *d*-glucuronic acid but it has not been identified with certainty. The methoxyl is ether-linked to the uronic acid but its position is not known. Some *d*-glucose is usually present along with the *d*-xylose among the products of hydrolysis of the hemicelluloses that give a coloration with iodine. O'Dwyer (2) states that these anhydroglucose units form a part of the hemicellulose molecule. There is some evidence for her statement (1). However, the *d*-glucose may come from traces of starch or dextrin present as an impurity. A hemicellulose from compression wood of white pine gave some *d*-mannose along with the *d*-xylose. A hemicellulose from normal wood of white pine gave both *d*-glucose and *d*-mannose together with *d*-xylose (3). Many plant mucilages (4), plant gums (5), and hemicelluloses (6) contain two or more different sugars in the molecule (7). It is not surprising therefore that some hemicelluloses from hardwoods contain *d*-glucose along with the *d*-xylose, especially in the early stages of their formation.

No attempt has been reported to determine the molecular size of hemicelluloses by viscosity, ultracentrifuge, or x-ray measurements. If each hemicellulose molecule contains one monomethoxyuronic acid, it is possible to estimate the length of the chain from the percentage of either the carbon dioxide or the methoxyl. However, this method is open to the same criticism that has been made of end-group studies in calculation of the size of the cellulose and starch molecules.

O'Dwyer (2) reported the isolation from oak wood of a soluble polyuronide consisting of a monomethoxyuronic acid combined with 6 molecules of *d*-xylose. In general the hemicelluloses of hardwoods are mixtures of

molecules varying in size from approximately 7 to approximately 19 molecules of *d*-xylose combined with 1 molecule of a monomethoxyuronic acid (1). They seem to bear the same relation to each other that the dextrans bear to each other and to starch. The work of Sands and Gary (8) and of Sands and Nutter (9) agrees with this conception of the hemicellulose molecule.

It is not known whether the hemicellulose molecules in the cell wall are of uniform or variable size. The reagents used in isolating and purifying them may cause partial hydrolysis. However, it is known that some mucilages vary in molecular size (4). Also, inulin which is isolated at different seasons consists of varying mixtures of polyfructose molecules (10). These materials probably exist as mixtures in the plant, since water alone is used in their isolation. This suggests that the hemicelluloses may also vary in chain length, as they exist in the cell wall.

In hemicelluloses from hardwoods the aldehyde group of the uronic acid is joined by a glycosidic union to a hydroxyl group on *d*-xylose. The various units of *d*-xylose are joined to each other by similar glycosidic linkages. It is not known which hydroxyl group is involved in these linkages nor is it known whether the linkage is  $\alpha$  or  $\beta$ . However, during the hydrolysis of the hemicelluloses to aldobionic acids there is a gradual shift from strong levorotation to strong dextrorotation. Furthermore, while little is known as to the action of enzymes on the free hemicelluloses, malt diastase does not hydrolyze appreciably those from hardwoods. These facts indicate the presence of  $\beta$ -glycosidic linkages.

The hemicelluloses are not dissolved out of plant materials by water but after they have been dissolved by alkali and reprecipitated by an acid they are soluble to some extent in water. This suggests that they are joined by an ester linkage between the carboxyl group of the uronic acid and a hydroxyl group of some cell wall constituent. This hydroxyl group might be on lignin, cellulose, or another hemicellulose molecule. The last named structure would give rise to a long branching chain of hemicellulose molecules similar in a general way to the structure suggested for starch by Staudinger (11).

Other materials are usually dissolved out of plant products by alkali along with the hemicelluloses. Frequently these other materials seem to be combined with the hemicellulose and are left insoluble when the hemicellulose is hydrolyzed by acids. It seems probable that in the cell wall the hemicellulose molecule is attached at one end by an ester linkage with some cell wall constituent while at the other end it is attached by a glycosidic union with some other cell wall constituent, possibly lignin (12). When plant materials are treated with alkali, the ester linkage is broken and the alkali salt of the hemicellulose dissolves, carrying into solution with it the lignin or other material.

Any suggestion as to the origin of the hemicelluloses must take into account the occasional presence of *d*-glucose, *d*-mannose, and *d*-galactose along with the pentoses in the hemicellulose molecule. Furthermore the type of glycosidic linkage, whether  $\alpha$  or  $\beta$ , and the location of the oxygen bridge must be considered. The hemicelluloses of hardwoods might be formed from cellulose, starch, dextrin, or from simple sugars. The stability of cellulose suggests that it is not the source of the hemicelluloses. Starch, or more likely dextrin, is probably their source. If the primary alcohol groups of dextrin were oxidized to carboxyl, the resulting uronic acid could by decarboxylation give rise to hemicelluloses. Such a material coming from dextrin should be an  $\alpha$ -glycoside and should have a 1-4 oxygen linkage. It is a fact that the polyuronide hemicelluloses obtained from hardwoods approximate the size usually ascribed to the dextrans.

Living cells utilize simple sugars in the synthesis of innumerable compounds. Some organisms utilize sugars in the synthesis of polyuronides similar in their general structure to the hemicelluloses (13). It is quite possible that plants may synthesize hemicelluloses directly from *d*-glucose or *d*-galactose. In this case one might expect to find any of the common pentose and hexose sugars in the hemicellulose molecule. Furthermore there would be no limitation to the kind of linkage and to the position of the oxygen bridge.

#### EXPERIMENTAL

*Material Used*—In order to learn more about the composition, structure, and possible origin of hemicelluloses from dicotyledonous trees, the cottonwood, *Populus macdougalii*, was chosen for study. This tree is quite different from the oak, black locust, and other hardwoods previously studied. Branches, approximately 2 inches in diameter, which were known to be the growth of the previous season, were cut in late December and allowed to dry in the air. The bark was removed and the wood converted to a powder.

*Extraction of Hemicelluloses and Pectic Materials*—The powdered wood was thoroughly extracted with acetone, hot ethanol, and hot water. The extraction with hot water was continued until the extract no longer gave the iodine test for starch.

The hemicelluloses and pectic materials were isolated, purified, and fractionated before and after chlorination of the wood, as described in previous publications (1). The total yield of the purified hemicellulose was 10 per cent of the weight of the wood. A later extraction gave a much higher yield of the hemicellulose. The total yield of the purified pectic material was 1.5 per cent of the weight of the wood. All of the hemicelluloses and pectic materials isolated before chlorination of the wood gave a positive

test for starch. None of those isolated after chlorination of the wood gave any test for starch.

*Fractionation and Analysis of Hemicelluloses*—Four fractions of the hemicellulose were obtained and analyzed. Fractions A and C were the water-insoluble hemicelluloses obtained respectively before and after chlorination of the wood. Fractions B and D were the water-soluble portions obtained respectively before and after chlorination of the wood. Fractions A and B were further separated into Fractions A<sub>1</sub> and A<sub>2</sub>, B<sub>1</sub> and B<sub>2</sub>, in which A<sub>1</sub> and B<sub>1</sub> are the less soluble portions while A<sub>2</sub> and B<sub>2</sub> are the more soluble portions. Fractions C and D were not further separated. In Table I are given the analytical results obtained on Fractions A<sub>1</sub>, B<sub>1</sub>, C, and D. Fractions A<sub>2</sub> and B<sub>2</sub> gave results intermediate between those of Fractions A<sub>1</sub> and B<sub>2</sub> and these results are not included in Table I.

TABLE I  
*Analyses of Hemicelluloses Obtained from Cottonwood*

	Fraction A <sub>1</sub>	Fraction B <sub>1</sub>	Fraction C	Fraction D
Carbon dioxide, %.....	3.12	4.15	3.08	3.42
Methylated uronic acid, %.....	14.70	19.57	14.47	16.12
Xylan, %.....	83.90	78.30	83.90	81.10
Total, %.....	98.60	97.87	98.37	97.22
Methoxyl, %.....	2.69	3.70	2.25	2.48
$[\alpha]_D^{25}$ , degrees.....	-70.8	-53.0	-73.4	-64.2
Equivalent weight.....	1410	1060	1428	1287
Xylan units.....	9.1	6.5	9.2	8.1
Starch test.....	+	+	-	-

*Discussion of Analytical Results*—The analytical results indicate that the hemicelluloses from cottonwood are smaller than those from woods previously studied (1). This would seem to indicate that hemicelluloses from different woods really vary in size. There are other possible explanations, such as difference in age of the wood and possible seasonal variation. The fact was noticed again that the hemicelluloses obtained after chlorination of the wood were not colored by iodine, while those obtained before chlorination were so colored. This may be because the material giving the color is an impurity which is removed during chlorination. However, it may be that the surface layers of hemicellulose are in a transition stage and still have some starch attached, while the deeper layers of hemicellulose no longer contain any glucose units.

*Hydrolysis of Hemicelluloses*—Weighed amounts of the hemicelluloses obtained before and after chlorination of the wood were hydrolyzed sepa-

rately and the sugars and barium salts were isolated as previously described (1). Large amounts of crystalline *d*-xylose and small amounts of crystalline *d*-glucose were obtained from Fractions A and B. Crystalline *d*-xylose was the only sugar obtained from Fractions C and D. The *d*-xylose was identified by its  $[\alpha]_D^{25} = +18.5^\circ$  and by Bertrand's reaction (14). The *d*-glucose was identified by conversion to saccharic acid (15).

The barium salt obtained by hydrolysis of Fractions A and B was analyzed with the following results: 8.12 per cent carbon dioxide, 5.66 per cent methoxyl,  $[\alpha]_D^{25} = +67.5^\circ$ . The theoretical percentages for a barium salt of a monomethoxyuronic acid combined with 2 xylan units would be 8.15 per cent carbon dioxide and 5.74 per cent methoxyl.

*Pectic Materials*—The pectic materials obtained before and after chlorination of cottonwood were combined, purified, and analyzed as described in previous publications (16). The presence of large amounts of *d*-galacturonic acid in the purified pectic material was established by the method of Heidelberger and Goebel (17). The pectic material had the general physical and chemical properties of pectic acid. On analysis it gave the following results: 18.5 per cent carbon dioxide, 19 per cent furfural,  $[\alpha]_D^{25} = +216^\circ$ . When it was dissolved in dilute ammonium hydroxide, filtered, and acidified with acetic acid, and calcium chloride solution added, it gave calcium pectate, insoluble in large amounts of boiling water.

#### SUMMARY

Previous work bearing on the composition, structure, and origin of the polyuronide hemicelluloses of hardwoods is reviewed. The isolation of hemicelluloses and pectic materials from cottonwood is described. These hemicelluloses form a mixture of molecules consisting of a methoxyuronic acid combined with a chain of between approximately 7 and 9 xylan units. These hemicelluloses are smaller than those previously described. Those obtained before chlorination of the wood gave the iodine test for starch, while those obtained after chlorination of the wood did not give this test. After hydrolysis of the hemicelluloses, a barium salt was isolated which consisted of a methoxyuronic acid combined with 2 molecules of *d*-xylose. The composition of these hemicelluloses suggests that they originate by partial oxidation and decarboxylation of starch or dextrin. Pectic materials were isolated that appear to be identical with pectic acid.

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# ELECTROLYTE AND WATER EXCHANGE BETWEEN SKELETAL MUSCLE AND PLASMA IN THE DOG FOLLOWING ACUTE AND PROLONGED EXTRACELLULAR ELECTROLYTE LOSS\*

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A number of studies have recently been reported (1-8) to indicate that osmotic equilibrium between the intracellular and extracellular compartments of skeletal muscle is attained chiefly by a transfer of water. Since, in the normal animal, the composition of the intracellular fluids remains relatively constant, it is believed that the concentration of sodium in the extracellular fluids plays a prominent rôle in the exchanges of water between the two compartments. Thus, a loss of extracellular electrolyte (sodium) with little change in total body water leads to an increase of intracellular water, while a gain of extracellular electrolyte (sodium) with little change in total body water leads to a decrease of intracellular water. On the other hand, a gain or loss of extracellular electrolyte of isotonic concentration leads to an increase or decrease of the extracellular volume with little or no exchange of water between the extracellular and intracellular compartments.

The experiments included in this paper were undertaken for the following purposes: (1) to observe the electrolyte and water exchanges between the extracellular and intracellular compartments of skeletal muscle accompanying an acute loss of extracellular electrolyte, with little change in total body water, and to observe if any subsequent exchanges occur when the loss of extracellular electrolyte is allowed to persist for reasonably prolonged periods, and (2) to observe the electrolyte and water exchanges between the extra- and intracellular compartments of skeletal muscle which accompany the simultaneous loss of extracellular electrolyte and water, induced by the loss of the gastric secretions through vomiting as the result of pyloric obstruction.

## *Methods and Calculation*

Adult male dogs were used in this study and were placed on a constant diet (the diet suggested by Nilson (9) supplemented by 5 gm. of NaCl per

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day) for a period of at least 7 days before the experiments were undertaken. The control blood was withdrawn under oil from the femoral artery 24 hours after the last feeding and placed under oil in a special centrifuge tube containing heparin to prevent clotting. The sample was then centrifuged immediately for the plasma analyses. Skeletal muscle (lumbar portion, sacrospinalis) was obtained under sodium pentobarbital anesthesia as quickly as possible following the collection of the blood and was sampled for the several analyses in the manner previously described (6).

After the animals had been allowed to recover from the control muscle biopsy (4 to 6 days), the loss of extracellular electrolyte was induced in the following ways. In the first group of experiments an acute loss of extracellular electrolyte with little change in total body water was produced by the familiar procedure (1) of injecting 100 cc. of 5 per cent glucose solution per kilo of body weight intraperitoneally and subsequently removing a volume of fluid equal to that injected  $4\frac{1}{2}$  hours after the injection. The experimental arterial blood and skeletal muscle samples were obtained in one series of animals immediately after the removal of the peritoneal fluid. In a second series, the animals were maintained for periods of 5 to 6 days following the removal of the peritoneal fluid on a diet with a very low sodium chloride content and distilled water *ad libitum*, or no food and distilled water *ad libitum*, before the experimental arterial blood and skeletal muscle samples were obtained. In the second group of experiments a simultaneous loss of extracellular electrolyte and water was produced by the loss of the gastric secretions through vomiting as the result of obstructing the pylorus by a surgical tape ligature. Experimental arterial blood and skeletal muscle samples were obtained at the intervals indicated in Table III.

The following determinations were carried out on the plasma: water, chloride, sodium, and potassium; on the skeletal muscle: water, chloride, sodium, potassium, total neutral fat, and total nitrogen. The chemical methods were the same as those employed in previous papers (6, 10), except that the potassium of plasma and muscle was determined on the aqueous solution of the ash by adapting the photoelectric method of Tenery and Anderson (11).

The volumes of the extracellular (*F*) and intracellular (*C*) phases of muscle were calculated in the manner outlined by Hastings and Eichelberger (2), which is based on the assumption that all the chloride is extracellular and is present at a concentration equal to that of an ultraniltrate of plasma. In order to approximate the volume changes produced in the extra- and intracellular phases of 1 kilo of original muscle, the calculations were made relative to the control series, assuming a constant solid of the intracellular phase.<sup>1</sup>

<sup>1</sup> Collagen nitrogen determinations were also carried out on the skeletal muscle samples by the method of Spencer, Morgulis, and Wilder (12). These data have



The partition of electrolytes (sodium and potassium) between the extra- and intracellular phases of muscle was carried out as described by previous authors (2, 14). That is, the extracellular electrolyte per kilo of muscle equals the product of the volume of extracellular water and the concentration of electrolyte in extracellular water, while the intracellular electrolyte per kilo of muscle equals the difference between the total electrolyte and the electrolyte located in the extracellular phase. The experimental values herein reported for intracellular sodium and potassium are given in terms of a kilo of intracellular water and have been corrected to an intracellular solid content equal to that of the control.

#### EXPERIMENTAL

*Control Observations*—A summary of the data obtained from the analysis of plasma and of skeletal muscle from seventeen normal dogs is given in Table I. These data are in good agreement with those previously reported from this laboratory (6, 10). It might be pointed out, however, that slightly higher mean values for sodium, potassium, and water of skeletal muscle were obtained in the present series.

*Acute Loss of Extracellular Electrolyte, with Little Change in Total Body Water. Immediate Changes*—The results of the analysis of plasma and of skeletal muscle from five dogs, obtained immediately after the removal of the peritoneal fluid ( $4\frac{1}{2}$  hours after the injection of 5 per cent glucose solution), are presented in Table II. By comparing these data with those given in Table I, it will be observed that there was a fall in the plasma chloride and sodium concentrations and a decrease in the plasma water content. Similarly the skeletal muscle chloride and sodium contents also became lowered; however, the muscle water remained essentially unchanged. The mean potassium figures for plasma and skeletal muscle were slightly below the controls, but the changes are probably insignificant.

With the acute loss of extracellular electrolyte there was a decrease in the extracellular phase ( $F$ ) and a gain of intracellular water  $\{H_2O\}_c$ . Thus, before injection, the normal extracellular phase ( $F$ ) of 1 kilo of skeletal muscle amounted to 158 gm., with a standard deviation ( $\sigma$ ) of  $\pm 15$  gm. (Table I). Immediately after the removal of the peritoneal fluid the mean values of ( $F$ ) and  $\{H_2O\}_c$  amounted to 115 and 738 gm., respectively. It will be observed that the change produced in the bulk of 1 kilo

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not been included in the present paper nor have the calculations of the extracellular phase (connective tissue phase) based on the connective tissue content. This omission was made for the reason that the same conclusions could be drawn whether or not the connective tissue content was taken into account. In addition, the recent report by Lowry, Gilligan, and Katersky (13), indicating that the method of Spencer, Morgulis, and Wilder yields variable high results, makes it desirable to redetermine the collagen nitrogen content of normal dog skeletal muscle by the procedure described by the former authors.

of muscle amounted to an average increase of 13 gm. per kilo of control muscle,  $\Delta M$ , consisting of an average 54 gm. increase in the intracellular phase,  $\Delta C$ , and an average 41 gm. decrease in the extracellular phase,  $\Delta F$ .

The partition of sodium and potassium between the extra- and intracellular compartments of skeletal muscle revealed that in normal muscle (Table I) the "excess" sodium which can be located in the intracellular phase,  $\{Na\}_c$ , amounted to 12.7 milliequivalents,  $\sigma \pm 4.7$  milliequivalents, per kilo of intracellular water, while the intracellular potassium per kilo

TABLE I

*Electrolyte and Water Content of Plasma and Skeletal Muscle of Seventeen Normal Dogs*

	Plasma				Muscle*			
	Mean	Maximum	Minimum	$\sigma$	Mean	Maximum	Minimum	$\sigma$
Chloride, <i>m.eq.</i> †	120.5	125	115	2.9	19.8	24	16	2.0
Sodium, <i>m.eq.</i> †	156.3	162	146	3.5	31.1	34	22	3.1
Potassium, <i>m.eq.</i> †	3.1	5	2	0.6	88.5	98	78	6.0
Water, <i>gm.</i> ‡	918.4	937	905	7.1	764.0	782	750	8.4
Extracellular phase, ( <i>F</i> ), <i>gm.</i>					158	182	133	15
Intracellular water, ( $H_2O$ ) <sub>c</sub> , <i>gm.</i>					608	630	582	14
Intracellular water, ( $H_2O$ ) <sub>c</sub> , <i>gm.</i> §					722	744	709	10
Intracellular sodium, $\{Na\}_c$ , <i>m.eq.</i>					12.7	20	1	4.7
Intracellular potassium, $\{K\}_c$ , <i>m.eq.</i>					145.0	162	128	11.7

\* Lumbar portion, sacrospinalis.

† Plasma values are expressed in terms of 1000 gm. of water and muscle values in terms of 1000 gm. of fat-free muscle.

‡ Plasma water is expressed as gm. per kilo.

§ Per 1000 gm. of muscle cells.

|| Per 1000 gm. of intracellular water.

of intracellular water,  $\{K\}_c$ , amounted to 145.0 milliequivalents,  $\sigma \pm 11.7$  milliequivalents. In the animals studied following the acute loss of extracellular electrolyte the amount of sodium which could be located in the intracellular phase became reduced. The mean value of  $\{Na\}_c$  was found to be 4.4 milliequivalents, with a range of from 13 to 0 milliequivalents. (While the lowest value of  $\{Na\}_c$  is indicated as 0, actually, in two animals, the "excess" sodium was slightly negative.) The mean intracellular potassium,  $\{K\}_c$ , was found to be 141.9 milliequivalents, which is only slightly below the mean control value.

From these results it would appear that, in normal dogs, an acute loss of extracellular electrolyte with little change in total body water is accom-

panied by a transfer of water from the extracellular to the intracellular compartment of skeletal muscle. Further, such a loss of extracellular electrolyte and exchange of water may be associated with a decrease in the amount of "excess" sodium which can be located in the intracellular phase and with little or no change in the amount of intracellular potassium.

TABLE II

*Electrolyte and Water Content of Plasma and Muscle from Dogs Following Intraperitoneal Injection of 5 Per Cent Glucose*

	Five dogs 4½ hrs. after injection						Seven dogs 5 or 6 days after injection					
	Plasma			Muscle*			Plasma			Muscle*		
	Mean	Maximum	Minimum	Mean	Maximum	Minimum	Mean	Maximum	Minimum	Mean	Maximum	Minimum
Chloride, m.eq.†.....	100.9	102	100	12.1	15	10	107.4	114	92	13.3	15	12
Sodium, m.eq.†.....	140.1	143	134	18.0	21	13	149.2	156	130	22.8	27	17
Potassium, m.eq.†.....	3.0	4	2	85.3	92	80	3.7	5	2	90.6	107	67
Water, gm.‡.....	889.9	900	881	767.5	770	762	909.3	918	905	765.3	774	753
Extracellular phase, (F), gm.....				115	139	93				119	132	109
Intracellular water, (H <sub>2</sub> O) <sub>c</sub> , gm.....				654	670	632				648	658	629
Intracellular water, {H <sub>2</sub> O} <sub>c</sub> , gm.§.....				738	742	734				736	744	724
Intracellular sodium, {Na} <sub>c</sub> , m.eq.  .....				4.4	13	0				9.7	17	3
Intracellular potassium, {K} <sub>c</sub> , m.eq.  .....				141.9	154	135				149.0	175	108
ΔM, gm. per kg.....				13	28	-10				4	44	-46
ΔC, " " ".....				54	63	38				42	74	8
ΔF, " " ".....				-41	-65	-15				-38	-54	-29

\* Lumbar portion, sacrospinalis.

† Plasma values are expressed in terms of 1000 gm. of water and muscle values in terms of 1000 gm. of fat-free muscle.

‡ Plasma water is expressed as gm. per kilo.

§ Per 1000 gm. of muscle cells.

|| Per 1000 gm. of intracellular water and corrected to an intracellular solid content equal to that of the control.

*Maintained Extracellular Electrolyte Loss*—The data obtained on seven dogs maintained for periods of 5 or 6 days, after the removal of the peritoneal fluid, on a diet very low in sodium chloride content, or with no food, and water *ad libitum*<sup>2</sup> are also given in Table II. By comparing the plasma

<sup>2</sup> Since no significant differences were observed between the animals allowed food and those in which food was withheld, the data have been grouped together.

and skeletal muscle changes encountered in these animals with those of the preceding experiment, a marked similarity will be noted. Thus, although the several values tended to return to normal, deficits of the plasma chloride and sodium concentrations, of the plasma water content, and of the skeletal muscle chloride and sodium still persisted. Further, the water content of the skeletal muscle remained unchanged. The mean potassium figures for plasma and skeletal muscle were slightly greater than the mean levels found in the controls; however, these changes are probably without real significance.

In the animals with maintained extracellular electrolyte loss, there was a persistence in the decrease of the extracellular phase, ( $F$ ), and in the gain of the intracellular water  $\{H_2O\}_c$ . Thus, the mean values of ( $F$ ) and  $\{H_2O\}_c$  were found to be 119 gm. per kilo of muscle and 736 gm. per kilo of muscle cells, respectively. The change produced in the bulk of 1 kilo of muscle amounted to an average increase of 4 gm. per kilo of control muscle,  $\Delta M$ , consisting of an average 42 gm. increase in the intracellular phase,  $\Delta C$ , and an average 38 gm. decrease in the extracellular phase,  $\Delta F$ . It will be noted that these calculated changes are essentially the same in magnitude as those encountered in the animals which were studied immediately after the removal of the peritoneal fluid.

The partition of sodium and potassium between the extra- and intracellular compartments of the skeletal muscle in the two groups of animals revealed that, on the average, there was a greater "excess" of sodium which could be located in the intracellular phase of the animals with maintained extracellular electrolyte loss. In the latter animals the average intracellular sodium was found to be 9.7 milliequivalents per kilo of intracellular water, while in the animals studied immediately after the removal of the peritoneal fluid the average figure was 4.4 milliequivalents. In the animals with maintained extracellular electrolyte loss, the average intracellular potassium was found to be 149.0 milliequivalents per kilo of cell water. Although the latter figure is somewhat higher than the average figure of 145.0 milliequivalents obtained in the control series, the difference is of questionable significance.

From these results it would appear that, in normal dogs, the gain of skeletal muscle cell water which accompanies an acute loss of extracellular electrolyte with little change in total body water may persist for reasonably prolonged periods, provided extracellular electrolyte replacement is prevented. Further, with such a prolonged increase in cell water of the skeletal muscle, there may be little or no change in the amount of intracellular potassium.

*Simultaneous Loss of Extracellular Electrolyte and Water during Pyloric Obstruction*—Data for six dogs with pyloric obstruction are presented in Table III. It will be seen that, with the exception of Dog C-4, there was

TABLE III  
Electrolyte and Water Content of Plasma and Muscle from Dogs with Pyloric Obstruction

	Plasma						Muscle*					
	Dog C-1	Dog C-2	Dog C-3	Dog C-4†	Dog C-5	Dog C-6	Dog C-1	Dog C-2	Dog C-3	Dog C-4†	Dog C-5	Dog C-6
Hrs. postoperative.....	48	48	24	48	48	72	48	48	24	48	48	72
Chloride, <i>m.eq.</i> ‡.....	108.1	97.5	107.3	123.8	109.5	80.4	15.7	11.1	15.1	16.8	17.8	10.8
Sodium, <i>m.eq.</i> ‡.....	172.5	152.8	147.8	169.8	152.8	139.1	26.5	16.1	23.8	24.5		
Potassium, <i>m.eq.</i> ‡.....	4.4	3.6	3.2	5.2	2.8	3.9	107.8	83.3	89.4	113.1		
Water, <i>gm.</i> §.....	889.0	893.0	889.0	889.2	905.0	894.0	752.0	761.0	773.0	743.0	751.0	758.5
Extracellular phase, (F), <i>gm.</i> .....							139	109	135	130	156	130
Intracellular water, (H <sub>2</sub> O) <sub>c</sub> , <i>gm.</i> .....							614	653	639	614	597	630
Intracellular water, [H <sub>2</sub> O] <sub>c</sub> , <i>gm.</i>   .....							713	733	738	707	708	724
Intracellular sodium, [Na] <sub>c</sub> , <i>m.eq.</i> ¶.....							6	0	8	5		
Intracellular potassium, [K] <sub>c</sub> , <i>m.eq.</i> ¶.....												
Δ <i>M</i> , <i>gm. per kg.</i> .....							168	135	152	170		
Δ <i>C</i> , " " ".....							-49	-15	39	-83	-53	-24
Δ <i>F</i> , " " ".....							-24	35	56	-45	-43	7
							-25	-50	-17	-38	-10	-31

\* Lumbar portion, sacrospinalis.

† Dog C-4 was not allowed water and there was comparatively little vomiting.

‡ Plasma values are expressed in terms of 1000 gm. of water and muscle values in terms of 1000 gm. of fat-free muscle.

§ Plasma water is expressed as gm. per kilo.

|| Per 1000 gm. of muscle cells.

¶ Per 1000 gm. of intracellular water and corrected to an intracellular solid content equal to that of the control.

a fall of the plasma chloride concentration, reaching the lowest level of 80.4 milliequivalents in Dog C-6. In connection with Dog C-4 an attempt was made to decrease the rapidity of dehydration by withholding fluid; however, the blood and skeletal muscle samples were taken for analysis the 2nd day postoperative, since the animal appeared critically ill. The plasma water content fell in each experiment, reaching levels found in the animals with acute extracellular electrolyte loss. Despite the loss of total extracellular electrolyte from the body, the concentration of sodium in the plasma tended to be maintained, which is in harmony with previous experimental and clinical findings. In two instances (Dogs C-1 and C-4) the plasma sodium concentration was actually found to be in excess of normal. In three animals (Dogs C-2, C-3, and C-5), a small plasma sodium deficit was encountered. However, when the loss of electrolyte and water was more prolonged (Dog C-6), an appreciable fall in the plasma sodium concentration occurred.

Although the electrolyte and water changes of the skeletal muscles were not entirely consistent, the muscle chloride tended to be reduced in each experiment and the greatest deficits were found in the animals (Dogs C-2 and C-6) with the lowest plasma chloride concentrations. Likewise, the muscle sodium tended to be lowered in the four animals in which the analyses were carried out, reaching an unusually low level in Dog C-2. The determinations of skeletal muscle potassium which were carried out on the same four animals revealed elevated levels in two animals (Dogs C-1 and C-4) and values within the normal range of variation in the remaining two animals. The skeletal muscle water appeared to be reduced in Dog C-4, to be somewhat below the control level in Dogs C-1 and C-5, and to be increased in Dog C-3.

With a simultaneous loss of extracellular electrolyte and water of isotonic concentration, a contraction of the extracellular phase of skeletal muscle, with little or no exchange of water between the extra- and intracellular compartments, may be anticipated. Under these circumstances the changes of the phases of 1 kilo of muscle relative to the control should be represented by a decrease in the bulk of the muscle attributable solely to a decrease in the bulk of the extracellular phase. From the data recorded in Table III, it will be observed that in agreement with the above interpretation  $\Delta F$  was negative in each experiment. Likewise, with the exception of Dog C-3, which had an unexplainably high skeletal muscle water content,  $\Delta M$  was also found to be negative. It will be noted, however, that changes in the bulk of the intracellular phase ( $\Delta C$ ) were also encountered. In certain of the experiments the direction of the changes in the bulk of the intracellular phase can be quite satisfactorily explained, since in no instance was there a removal of extracellular electrolyte and water of isotonic con-

centration. Thus, on the basis of the plasma sodium concentration and the plasma water content, it would appear that in Dogs C-1 and C-4 the condition is comparable to the removal of a hypotonic solution. Under such a circumstance the decrease in the bulk of the muscle should be accompanied by a decrease in the bulk of both the intracellular and extracellular phases. In accord with the latter interpretation it will be observed that in Dogs C-1 and C-4 the changes produced in the bulk of 1 kilo of muscle amounted to decreases of 49 and 83 gm. per kilo of control muscle, respectively, consisting in each case of decreases in the bulk of both the intracellular and extracellular phases. Similarly, on the basis of the plasma sodium concentration and the plasma water content, it would appear that in Dogs C-3 and C-6, and probably in Dogs C-2 and C-5, the condition is comparable to the removal of a hypertonic solution. If this were true, the decrease in the bulk of the muscle should be accompanied by a decrease in the bulk of the extracellular phase and a relatively smaller increase in the bulk of the intracellular phase. It will be observed that, in direction, such anticipated changes were realized in Dogs C-2 and C-6. Similarly, in Dog C-3 the direction of the changes in the bulk of both the extracellular and intracellular phases was in accord with the above interpretation. However, because of the unusually high skeletal muscle water content in this case, there was an increase in the bulk of the muscle, associated with a relatively large increase in the bulk of the intracellular phase and a smaller decrease in the bulk of the extracellular phase. It will be observed that in Dog C-5 the findings are not in accord with the anticipated changes, since the decrease in the bulk of the muscle was accompanied by a decrease in the bulk of the intracellular phase as well as in the bulk of the extracellular phase.

In the four animals in which it was possible to partition the sodium and potassium between the two compartments of muscle it was found that there was a tendency toward a decrease in the amount of "excess" sodium which could be located in the intracellular phase. There was an apparent gain in the amount of intracellular potassium in two animals (Dogs C-1 and C-4), a tendency toward a loss of intracellular potassium in one animal (Dog C-2), and little or no change of intracellular potassium in the remaining animal. It will be observed that there was no correlation between these changes of intracellular potassium and the amount of water which could be located in the intracellular phase.

#### DISCUSSION

The data herein reported offer further support to the view that the concentration of sodium in the extracellular fluids plays an important rôle in the exchange of water between the extra- and intracellular compartments

of skeletal muscle and that a transfer of water constitutes the principal adjustment in the maintenance of a uniform osmotic pressure. In a recent study Yannet and Darrow (7), experimenting with cats, critically examined the magnitude of the shifts of water which accompany changes in the concentration of extracellular electrolyte and the relation of these changes to alterations in the electrolyte pattern of the cell. Among other things these authors found that in the case of the skeletal muscle the shifts of water into

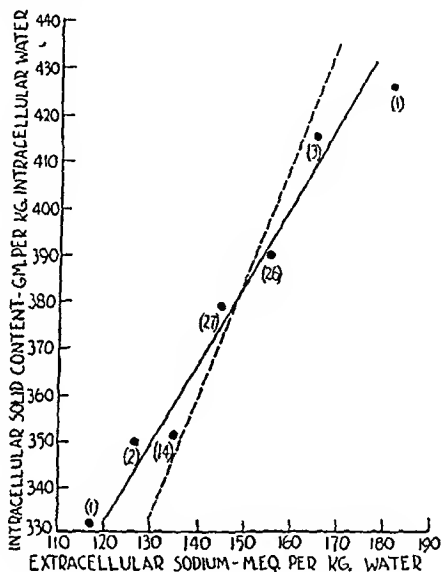


FIG. 1. Correlation between the extracellular sodium concentration and the intracellular solid content of skeletal muscle. The solid line was constructed by the method of least squares and the figures in the parentheses indicate the number of observations taken to compute the average intracellular solid content. The broken line indicates theoretical change, the calculations being made on the assumption of a constant intracellular potassium content.

cells in response to decrease in concentration of extracellular sodium were only about two-thirds that predicted by the assumption that the sole adjusting mechanism is a transfer of water between the extra- and intracellular compartments. In an attempt to make a similar evaluation in the case of the skeletal muscles of dogs, the extracellular sodium concentration and the intracellular solid content per kilo of intracellular water of the present series of animals and those previously reported<sup>3</sup> (6) were plotted

<sup>3</sup> Data from three animals, not reported, with external pancreatic fistula, two of which were given NaCl and NaHCO<sub>3</sub>, are also included.



against each other. Although such a plot revealed a definite trend, it must be frankly stated that there was a considerable scattering of points. However, when the average intracellular solid content per kilo of intracellular water for a given range of extracellular sodium concentration was plotted against the extracellular sodium concentration, the points fell along the solid line indicated in Fig. 1. The broken line was constructed for comparative purposes and represents calculated changes of the intracellular solid content with a change of the extracellular sodium concentration, on the assumption of a constant intracellular potassium content. It will be noted that the observed change in the intracellular solid content per unit change of extracellular sodium concentration is somewhat less than the calculated change. Although these data are admittedly not entirely satisfactory, it would appear, in agreement with the findings of Yannet and Darrow (7), that on the *average* the gain or loss of water by the skeletal muscle cells of dogs in response to a change in the extracellular sodium concentration is only about 60 to 70 per cent of that predicted by the assumption that osmotic adjustment is attained solely by a transfer of water between the extra- and intracellular compartments.

The comparatively small changes of the cellular potassium which were encountered in the animals with acute and prolonged extracellular electrolyte loss tend to indicate that under the conditions of the experiment the barrier separating the extra- and intracellular compartments of skeletal muscle remained relatively impermeable to potassium. In this connection Hamilton and Schwartz (15) were also unable to detect any change of cell potassium in skeletal muscle in dogs with uncomplicated dehydration, as produced by water deprivation. Similarly, Eichelberger was unable to demonstrate any retention of potassium in the skeletal muscle of normal dogs (8) and of dogs with a single hydronephrotic kidney (16) following increases in total body water and body potassium produced by the intravenous injection of large volumes of isotonic sodium chloride containing potassium. Such observations are of interest in view of the continually mounting evidence that the cellular membranes of skeletal muscle are permeable to potassium.

#### SUMMARY

A study has been made of the electrolyte and water exchanges between skeletal muscle and plasma in dogs (1) following the acute loss of extracellular electrolyte with little change of total body water, (2) following prolonged periods of extracellular electrolyte loss, and (3) following the simultaneous loss of extracellular electrolyte and water, produced by the loss of the gastric secretions through vomiting as the result of pyloric obstruction. The pertinent findings may be briefly summarized as follows.

1. An acute loss of extracellular electrolyte with little change of total

body water was found to be accompanied by a transfer of water from the extra- to the intracellular compartment of muscle. In addition, there was found to be a decrease in the amount of "excess" sodium which could be located in the intracellular phase and little or no change in the amount of intracellular potassium.

2. When extracellular electrolyte replacement was prevented, the hydration of the intracellular fluids and dehydration of the extracellular fluids which accompany an acute loss of extracellular electrolyte were found to persist for periods of 5 or 6 days. During this interval there was little or no change in the amount of intracellular potassium, while the average "excess" sodium tended to return to normal, accompanying a tendency toward the restoration of the plasma sodium concentration.

3. The data obtained on the animals following the simultaneous loss of extracellular electrolyte and water were not entirely consistent. However, in the majority of the experiments, the directions of the fluid exchanges in the muscles could be correctly predicted on the basis of the plasma sodium concentration and the plasma water content.

The results of the study were discussed briefly, with particular attention to the importance of the concentration of sodium in the extracellular fluids in controlling the exchange of water between the extra- and intracellular compartments of muscle and to the significance of the transfer of water in the maintenance of a uniform osmotic pressure.

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# CHANGES OF THE PLASMA VOLUME AND "AVAILABLE (THIOCYANATE) FLUID" IN EXPERIMENTAL DEHYDRATION\*

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As the result of a large amount of experimental work it is now well recognized that a close relation exists between the volume of water and the total amount of sodium in the extracellular compartment of the body. This being true, a loss of either water or sodium from the body is associated with a loss of the other constituent which, if continued, leads to symptoms of dehydration. On the basis of a view (1, 2) which has found general acceptance, it would appear that during the early stages of dehydration the losses of sodium and water are for the most part at the expense of the interstitial fluids, the volume and composition of the plasma thereby being protected. In other words, the plasma volume falls, with a resultant increase of the plasma protein concentration and of the hematocrit, only when the interstitial fluid volume has become depleted to a certain minimum. It is evident that if these conditions prevail, increasing levels of the plasma protein concentration and the hematocrit occur only after this critical loss is exceeded. In addition, when such changes do occur, they offer little aid in evaluating the extent of loss of total extracellular fluid. Such a situation would obviously be unfortunate from the clinical point of view, when an early detection of dehydration and information as to the extent of extracellular electrolyte and water loss are desired.

The experiments included in this paper were undertaken for the following purposes: (1) to obtain further information regarding the relation between the plasma volume and the "available (thiocyanate) fluid" and to ascertain the extent to which the volume of the plasma is sustained at the expense of the interstitial fluid in experimental dehydration, and (2) to evaluate the significance of changes of the plasma protein concentration and of the hematocrit in the detection of early and chronic dehydration.

## Methods

Adult male dogs were used in this study and were placed on a constant diet for a period of at least 1 week before the experiments were undertaken. After the control observations had been completed, dehydration was then

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produced in the following manner: (1) by injecting 100 cc. of a 5 per cent glucose solution per kilo of body weight into the peritoneal cavity and then subsequently removing a volume of fluid equal to that injected  $4\frac{1}{2}$  hours after the injection, (2) by permitting the loss of the pancreatic secretions through an external pancreatic fistula, (3) by inducing a loss of the gastrointestinal secretions through vomiting as the result of obstructing the gastrointestinal tract at various levels, and (4) by simple food and water deprivation.

The following determinations were carried out in the control and experimental periods, after food had been withheld for the previous 18 to 24 hours: the plasma volume and the "available (thiocyanate) fluid" by the direct method of Gregersen and Stewart (3) as adapted to the photoelectric colorimeter by Gibson and Evelyn (4), the total plasma protein concentration, in duplicate, by micro-Kjeldahl determination of 0.5 cc. of plasma, and the hematocrit, in quadruplicate and without dilution, in Van Allen (5) hematocrit tubes.

Plasma volume and "available (thiocyanate) fluid" determinations were carried out in a series of forty-six adult male dogs, with a range of body weight from 9.0 to 26.6 kilos. The plasma volume was found to range from 34 to 57 cc. per kilo and the "available (thiocyanate) fluid" from 212 to 370 cc. per kilo. As has already been pointed out by Gregersen and Stewart (3), there was found to be a general parallel between the values for the plasma volume and the "available (thiocyanate) fluid." For the series of forty-six dogs the mean of the ratio of "available (thiocyanate) fluid" to plasma volume was found to be 6.5, with a range of variation of from 5.3 to 7.9. Although the differences were not too impressive, it was found, as was also noted by the above workers, that the ratio tended to be greater in the animals in which the volumes per kilo were high. It is now generally appreciated that no uniformity of the volumes can be realized in a series of "normal" animals of varying age, sex, weight, or breed. Consequently, it is necessary to compare the volumes of the plasma and "available (thiocyanate) fluid" encountered experimentally with the respective control levels for each animal, in order to evaluate more clearly any change from normal.

#### EXPERIMENTAL

*Plasma Volume and "Available (Thiocyanate) Fluid" in Dehydration Following Intraperitoneal Injection of Glucose*—The results which were obtained on nine dogs before and following the intraperitoneal injection of 5 per cent glucose are given in Table I. In each instance a volume of fluid equal to that injected was removed  $4\frac{1}{2}$  hours after the injection. Four animals were studied immediately following the removal of the peritoneal fluid,

while the remaining five animals were maintained on a diet with a very low sodium chloride content, or no food, and water *ad libitum* for a period of 5 or 6 days before the experimental determinations were carried out.<sup>1</sup> From the data presented in Table I it will be seen that immediately following the removal of the peritoneal fluid there was a decrease in both the plasma volume and the "available (thiocyanate) fluid." In the case of the plasma volume the deficits amounted to from 30 to 37 per cent of the control volume, while those for the "available (thiocyanate) fluid" amounted to from 13 to 34 per cent of the control level. It will be observed, however, that

TABLE I

*Changes of Plasma Volume and "Available (Thiocyanate) Fluid" in Dogs Following Intraperitoneal Injection of 5 Per Cent Glucose*

100 cc. of glucose per kilo of body weight were injected and a volume of fluid equal to that injected was removed 4½ hours after the injection.

Dog No.	Control weight	Control volumes		Change of volume				Plasma protein		Hematocrit		Time after removal of peritoneal fluid
		Plasma	SCN	Plasma	SCN	Plasma	SCN	Control	Experimental	Control	Experimental	
	kg.	cc.	cc.	cc.	cc.	per cent	per cent	gm. per 100 cc.	gm. per 100 cc.	per cent	per cent	
A-3	19.2	922	5330	-331	-1700	-36	-32	7.88			52.3	Immediately
A-5	21.0	758	4450	-283	-1500	-37	-34	6.46	9.90	51.6	70.5	"
A-6	20.7	934	5530	-278	-710	-30	-13	7.21	9.97	43.0	51.5	"
A-7	22.3	759	4910	-264	-930	-35	-19	6.47	8.05	55.0	69.0	"
A-4	20.9	990	5980	-489	-950	-51	-16	5.99	7.76	50.8	57.6	5 or 6 days
A-8	22.9	934	6110	-260	-690	-28	-11	6.00	6.82	52.1	51.4	5 " 6 "
A-9	24.3	971	6400	-257	-1150	-26	-18	6.33	7.87	44.6	56.3	5 " 6 "
A-12	26.6	951	6520	-53	-750	-6	-12	7.25	7.70	54.8	53.0	5 " 6 "
A-13	22.0	934	6550	-136	-860	-15	-13	6.55	7.50	50.4	57.5	5 " 6 "

of the total loss of fluid from the extracellular compartment, the interstitial fluid (approximately equal to the "available (thiocyanate) fluid" minus the plasma volume) contributed a much larger amount.

The results obtained on the five animals with prolonged extracellular electrolyte loss are of some interest, since it may be anticipated that during a period of 5 or 6 days some adjustment between the plasma and the interstitial compartment might take place. That is, an adjustment might conceivably occur in the direction of a restoration of the plasma volume at the

<sup>1</sup> Certain of the animals listed in Table I, as well as those with pyloric obstruction given in Table II, were also included in the previous study (6) dealing with the electrolyte and water exchanges between skeletal muscle and blood in dehydration.

expense of the interstitial fluid. It will be observed that in the majority of the animals there appeared to be some restoration of the plasma volume, since the deficits of plasma volume in per cent of the control levels were

TABLE II

*Changes of Plasma Volume and "Available (Thiocyanate) Fluid" in Dogs with External Pancreatic Fistula and with Upper Intestinal Tract Obstruction*

Dog No.*	Control weight	Day post-operative	Control volumes		Change of volume				Plasma protein	Hemato-crit
			Plasma	SCN	Plasma	SCN	Plasma	SCN		
	kg.		cc.	cc.	cc.	cc.	per cent	per cent	gm. per 100 cc.	per cent
B-2	9.0	0	475	2900					6.11	38.9
		1							6.92	
		6							7.12	39.3
B-4	21.7	0	952	6020	-206	-1040	-43	-36	7.25	40.4
		1							6.62	46.9
		3							9.25	44.5
B-6	14.4	0	655	4350	-193	-1260	-20	-21	7.45	37.9
		2							9.82	41.6
		3							5.82	46.9
C-1	13.6	0	600	3760	-170	-1060	-26	-24	6.55	47.7
		1							7.71	55.4
		2							9.61	53.5
C-2	19.4	0	836	5250	-183	-1050	-30	-28	5.75	42.6
		1							7.86	48.7
		2							9.25	51.4
C-3	15.2	0	868	5380	-386	-1120	-46	-21	5.78	49.7
		1							8.70	59.1
		2							8.20	40.8
C-4	14.0	0	591	4050	-176	-560	-20	-10	9.45	45.2
		1							6.24	38.0
		2							7.30	44.2
C-5	23.8	0	935	5680	-53	-610	-9	-15	8.03	45.6
		1							6.50	47.5
		2							6.92	50.8
C-6	22.3	0	1148	6600	-150	-300	-16	-5	7.97	51.8
		1							6.86	42.4
		2							7.64	44.6
		1			-282	-480	-25	-7	8.02	45.3
		2							8.71	45.2
		3							9.23	45.3

\* Dogs B-2, B-4, and B-6 had external pancreatic fistulas and Dogs C-1 to C-6 had obstruction of the upper intestinal tract.

somewhat less than those encountered immediately following the removal of the peritoneal fluid. Similarly, however, there was also a tendency for the "available (thiocyanate) fluid" to return to normal. In these experi-

ments, therefore, little evidence was found to support the belief that the plasma volume is held at its normal level by adjustments in the interstitial compartment.

*Animals with External Pancreatic Fistula or with Pyloric Obstruction*—The results of a study of three dogs with dehydration produced by the loss of the pancreatic secretions through external pancreatic fistulas and of six dogs with dehydration produced by the loss of the gastric secretions by vomiting as the result of obstructing the pylorus are given in Table II. It will be observed that in every instance there was a fall of the plasma volume as well as of the "available (thiocyanate) fluid." As in the preceding experiments, it will be noted that of the total loss of fluid from the extracellular compartment, a larger amount was contributed by the interstitial fluid than by the plasma. However, the results again fail to demonstrate a maintenance of the plasma volume at the expense of the interstitial fluid during the course of dehydration.

*Animals with Intestinal Tract Obstruction at Various Levels and Animals Following Food and Water Deprivation*—In an attempt to produce a slower rate of dehydration, obstruction was produced at the following levels of the intestinal tract: upper jejunum, two animals; terminal ileum, four animals; and colon, three animals. In addition, food and water were withheld from four normal animals for periods of from 3 to 7 days. In Fig. 1 the per cent change of the plasma volume has been plotted against the per cent change of the "available (thiocyanate) fluid." The changes observed in the previous groups of animals have also been included for comparative purposes. The solid line was constructed to indicate equal per cent changes of the two volumes.

It will be observed that in only one instance was the plasma volume completely sustained, with a fall of the "available (thiocyanate) fluid" (solid triangle indicating a change of the plasma volume of +4 per cent and a change of the "available (thiocyanate) fluid" of -22 per cent). This observation was obtained on an animal with an obstruction of the terminal ileum, the 4th day postoperative. On the previous day the same animal exhibited changes of the plasma volume and "available (thiocyanate) fluid" of -7 and -15 per cent, respectively. The three additional observations which were carried out during the succeeding 8 days revealed a progressive fall of the plasma volume with little further change of the "available (thiocyanate) fluid." In all of the remaining animals studied, some fall of the plasma volume was found to be associated with the decrease of "available (thiocyanate) fluid." It will be observed that a number of points fall to the left of the line, indicating some maintenance of the plasma volume at the expense of the interstitial fluid. However, the majority of the points fall to the right of the line. In addition, it will be observed that the plasma

volume was found to be reduced significantly in a number of the animals in which the "available (thiocyanate) volume" had become decreased by only 10 per cent or less.

*Plasma Protein and Hematocrit Changes in Dehydration*—The data which have been presented above lead to the conclusion that with an occasional exception a fall of the plasma volume constitutes an early change in experi-

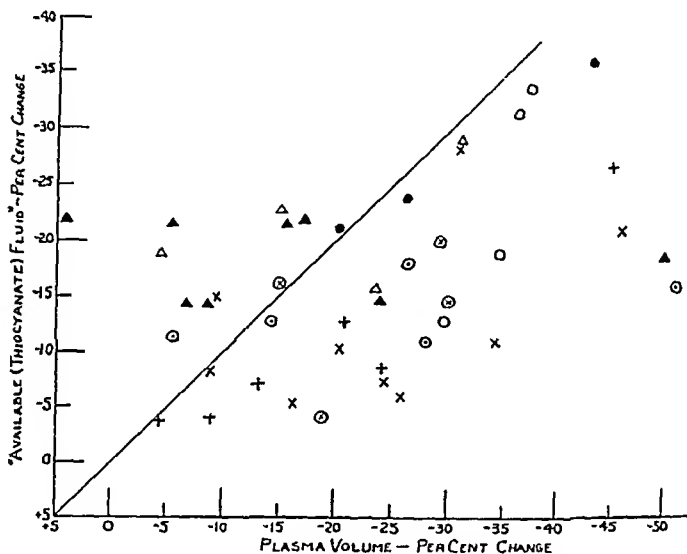


FIG. 1. Relation between the per cent change of the plasma volume and of the "available (thiocyanate) fluid" in experimental dehydration. The solid line represents equal per cent changes of the two volumes. The symbols indicate the following experiments: ○ immediately after the removal of peritoneal fluid following the intraperitoneal injection of glucose (4), ⊙ 5 or 6 days after the removal of peritoneal fluid (5), ● pancreatic fistula (3), × obstruction of the pylorus (6), Δ obstruction of the upper jejunum (2), ▲ obstruction of the terminal ileum (4), + obstruction of the colon (3), and ⊗ food and water deprivation (4), where the numbers within the parentheses indicate the number of animals.

mental dehydration. If this is true, an increase of the plasma protein concentration and of the hematocrit should also be encountered in the early stages of dehydration. From the data presented in Table II it will be seen that in every instance the first determination of the plasma protein concentration carried out during the course of dehydration showed some elevation above the control level. Further, from the data presented in Tables I and II, it will be noted that during the periods of more prolonged dehydration



the plasma protein concentration continued to indicate qualitatively a plasma volume deficit. However, from the point of view of a quantitative indication of the plasma volume decrease, the changes of the plasma protein concentration would not appear to be as consistent. Thus, the fall of the plasma volume was calculated on the basis of the change of the plasma protein concentration from the control level and compared with the plasma volume deficit measured directly. In the animals studied immediately following the acute loss of extracellular electrolyte (Table I), the calculated change was found to amount on the average to 91 per cent of the observed change. However, a similar calculation applied to the plasma protein figures in the five dogs with a maintained extracellular electrolyte loss revealed that the calculated change amounted on the average to only 71 per cent of the observed change, with a range of variation from 43 to 104 per cent. Likewise the plasma protein changes encountered in the experiments recorded in Table II failed to reflect accurately the plasma volume changes as measured directly. Attention might be called here to a finding, which will be discussed in more detail in a subsequent publication, that with considerably prolonged periods of dehydration the plasma protein concentration may actually fall to the control level or below, despite a rather marked deficit in plasma volume. For the sake of illustration, an animal studied for a period of 14 days following an obstruction of the colon showed a progressive decrease of the plasma volume, the maximum deficit amounting to 35 per cent of the control value. Despite the fall of plasma volume the plasma protein concentration decreased progressively from a control level of 7.32 gm. to 5.37 gm. per 100 cc. From this, and other experiments, it would appear that inadequate food intake during prolonged periods of dehydration may have a pronounced effect upon the total circulating plasma protein.

From the data presented in Table II it will be seen that with the exception of Dog B-4, the hematocrit also showed some increase above the control level in the early stages of dehydration. It would appear, however, that the changes of the hematocrit are equally as unreliable as those of the plasma protein concentration in detecting both qualitatively and quantitatively the plasma volume deficits in the animals studied with prolonged periods of dehydration. Thus in Dogs A-8 and A-12 (Table I) with maintained extracellular electrolyte loss, the hematocrit readings were found to be below the control levels despite a reduction of the plasma volume. A similar situation was encountered in the case of Dog B-4 (Table II), while in Dogs B-2 and C-6 it is immediately evident that the increases of the hematocrit and deficits of the plasma volume are not comparable. In general, the changes of the plasma volume calculated on the basis of the hematocrit exceeded the observed deficits in the animals with acute extracellular

electrolyte loss. In the remaining animals considerable variability was noted; however, most frequently, the calculated changes of the plasma volume were somewhat less than the observed deficits.

#### DISCUSSION

From his studies Gamble (1, 2) has come to the conclusion that with the loss of extracellular fluid during the course of dehydration the volume of the plasma tends to be maintained at the expense of the interstitial fluid. If this is true, the plasma volume can be expected to fall only after the interstitial fluid volume has become reduced to a certain minimum. That the interstitial fluid does serve to sustain the plasma volume is reconfirmed in the present experiments, since, of the total losses of the extracellular fluid from the body, the losses of interstitial fluid were always found to be greater than those of the plasma. However, a maintenance of the plasma volume at the expense of the interstitial fluid was found to be only an occasional exception in the types of experimental dehydration studied. In other words, it would appear that the plasma as well as the interstitial fluid contributes to the extracellular fluid losses in the early stages of dehydration. Gregersen and Stewart (3) were likewise led to this conclusion, since their studies also failed to indicate that the volume of the plasma is independent of the quantity of interstitial fluid even with moderate changes in the volume of the extracellular fluid.

If a decrease in the volume of the plasma occurs in the early stages of dehydration, then increases of the plasma protein concentration and of the hematocrit should also become evident in the early stages of dehydration. This was found to be true in the present experiments with only a few exceptions. It would appear, therefore, that it is generally possible to detect qualitatively a deficit of the plasma volume in acute experimental dehydration from the changes of the plasma protein concentration or the hematocrit. However, from the point of view of a quantitative indication of plasma volume deficit the latter measurements are apparently not entirely reliable even in acute experimental dehydration. From the present studies it would appear that as the period of dehydration becomes more prolonged the changes of the plasma protein concentration and of the hematocrit may not only be entirely unsatisfactory in indicating quantitatively a decrease of plasma volume, but may also fail to indicate correctly the change qualitatively. Such discrepancies would appear to be most likely encountered when malnutrition becomes a complicating feature. In this connection attention might be called to the recent findings of several authors (7-10) indicating that it is not possible accurately to determine changes in the plasma volume from changes of the plasma protein concentration and the hematocrit.

It should be realized that the conclusions arrived at above are based upon the assumption that the changes of the plasma volume and "available (thiocyanate) fluid" from their respective control levels are correctly detected by the methods employed. Since identical procedures were carried out in the control and experimental periods, it would appear that the results obtained are adequate for the conclusions drawn.

#### SUMMARY

Determinations of the plasma volume, "available (thiocyanate) fluid," plasma protein concentration, and hematocrit were carried out in dogs before and following dehydration produced in several ways. From the results of the study on experimental dehydration the following conclusions are drawn.

1. Both the interstitial fluid and the plasma contribute to the fluid lost from the extracellular compartment in dehydration.

2. The interstitial fluid serves to sustain the volume of the plasma in the sense that of the total loss of fluid from the extracellular compartment a much larger amount is contributed by the interstitial fluid than by the plasma.

3. A deficit of the plasma volume occurs in the early stages of dehydration, a maintenance of the plasma volume at the expense of the interstitial fluid being only an occasional finding.

4. Changes of the plasma protein concentration and of the hematocrit are suitable for the qualitative detection of a plasma volume deficit in acute dehydration.

5. Frequently it is not possible accurately to determine changes of the plasma volume from changes of the plasma protein concentration and the hematocrit. This is particularly true following more prolonged periods of dehydration in which malnutrition constitutes a complicating feature. Under such circumstances, by determination of the plasma protein concentration and the hematocrit, a qualitative change of the plasma volume may fail to be detected accurately.

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